30863

NATIONAL LIBRARY
OTTAWA

CANADA

BIBLIOTHÈQUE NATIONALE
OTTAWA

NAME OF AUTHOR... GORDON.....LAWRENCE.....WEIR.....

TITLE OF THESIS..STUDIES...RELATED...TO...THE.....
ANTITUMOR..ANTIBIOTIC.....
MITOMYCIN...B...AND....MODELS.....

UNIVERSITY..OF...ALBERTA.....

DEGREE FOR WHICH THESIS WAS PRESENTED.....M.Sc.....

YEAR THIS DEGREE GRANTED....1976.....

Permission is hereby granted to THE NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed)..Gordon L. Weir.....

PERMANENT ADDRESS:

.424..35..ave...N.W..
.Calgary...alberta.....
.T2K..0S1.....

DATED...July..29.....1976

NL-91 (10-68)

INFORMATION TO USERS

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED

This copy was produced from a microfiche copy of the original document. The quality of the copy is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Canadian Theses Division
Cataloguing Branch
National Library of Canada
Ottawa, Canada K1A ON4

AVIS AUX USAGERS

LA THESE A ETE MICROFILMEE TELLE QUE NOUS L'AVONS RECUE

Cette copie a été faite à partir d'une microfiche du document original. La qualité de la copie dépend grandement de la qualité de la thèse soumise pour le microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

NOTA BENE: La qualité d'impression de certaines pages peut laisser à désirer. Microfilmee telle que nous l'avons reçue.

Division des thèses canadiennes
Direction du catalogage
Bibliothèque nationale du Canada
Ottawa, Canada K1A ON4

THE UNIVERSITY OF ALBERTA

STUDIES RELATED TO THE ANTITUMOR ANTIBIOTIC MITOMYCIN B AND MODELS

by

© GORDON LAWRENCE WEIR

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

FALL, 1976

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled .....
STUDIES RELATED TO THE ANTITUMOR ANTIBIOTIC MITOMYCIN B .....
AND MODELS .....
submitted by .GORDON.LAWRENCE.WEIR.....
in partial fulfilment of the requirements for the degree of
Master of SCIENCE.....

J W Town
.....
Supervisor
David R. Hopwood
.....
W M Bragg
.....

Date July 19 1976

To my wife, Pam,

for her steadfast devotion

and

To "Woody" Woods,

without whose support and encouragement

this thesis would not have been written

ABSTRACT

The base-catalyzed methanolysis of model monofunctional indoles was studied in order to gain information about the relative reactivity of 2- and 3-indolylcarbinyl systems, analogous to the proposed reactive sites of mitosanes which have been reductively activated. The leaving groups studied were also analogous to those found in the mitomycins. Reaction kinetics were studied by the method of nmr spectroscopy at a number of temperatures, thereby allowing the calculation of activation parameters.

The interaction of mitomycin B with DNA was examined using rapid and convenient ethidium fluorescence assays. Studies on the pH dependence of covalent cross-linking and alkylation of DNA by mitomycin B and its aziridine-ring-opened derivative have confirmed the involvement of both the aziridine and carbamate groups in the covalent attachment to DNA. In conjunction with the pH studies the pKa of mitomycin B was determined. By using a number of DNA's and variations of the ethidium assay it has been possible to partially define the DNA sites involved in covalent attachment.

It was found that mitomycin B induces single strand scission in DNA by the generation of superoxide and hydroxyl radicals; this scission is also produced by mitomycin B and mitomycin C when they are covalently bound to the DNA. The effect of intercalated ethidium on the scission produced by mitomycin B and mitomycin C was also investi-

gated.

Electroanalytical experiments with mitomycin B and its aziridine-ring-opened derivative yielded results consistent with the comparative reactivities found for mitomycin B and mitomycin C.

ACKNOWLEDGEMENTS

The author wishes to express sincere gratitude and appreciation to his research director, Professor J.W. Lown, for his encouragement, understanding and guidance throughout the progress of this work.

Sincere thanks are also extended to:

Mr. R.N. Swindlehurst and his associates for infrared and 60 MHz proton magnetic resonance spectra;

Dr. T. Nakashima and his associates for 100 MHz proton magnetic resonance spectra;

Dr. A. Hogg and his associates for mass spectra;

Mrs. D. Mahlow and her associates for elemental analyses;

Mr. C. Ediss for scintillation counting;

Dr. R.S. McDaniel for the use of the least-squares plotting program;

Mr. D. Johnson for DNA molecular weight determinations;

Dr. J.A. Plambeck and his associates for the electroanalytical experiments;

Mr. V. Ball for graphic work;

Mr. Ken Armstrong, P. Eng., for access to facilities for typing this manuscript;

Drs. F.F. Cantwell and D.L. Rabenstein for the use of special equipment;

Past and present members of the research group for helpful discussions and occasional supply of chemicals and

equipment, and especially to Dr. S.K. Sim for the experiments with PM2 CCC-DNA involving 22 and 24;

The National Research Council of Canada, the Province of Alberta, and the University of Alberta for financial support.

TABLE OF CONTENTS

CHAPTER		PAGE
I	INTRODUCTION	1
II	KINETIC STUDY OF 2- AND 3-INDOLYLCARBINYL COMPOUNDS	14
	A. Introduction	14
	B. 1-Methylgramine Methiodide 22	18
	1. Synthesis	18
	2. Product Runs	18
	3. Kinetic Study	21
	4. DNA Study	32
	C. 1-Methylisogramine methiodide 23	32
	1. Synthesis	32
	2. Product Runs	32
	3. Kinetic Study	34
	D. 1-Methylindol-2-ylmethyl N-*t*-butylcarbamate 24	42
	1. Synthesis	42
	2. Product Run	42
	3. Kinetic Study	42
	4. DNA Study	43
	E. Gramine Methiodide 26	50
	1. Synthesis	50
	2. Product Runs	50
	3. Kinetics	51
	4. DNA Studies	52
	F. Summary and Conclusions	55
	G. Experimental	62

TABLE OF CONTENTS (continued)

CHAPTER		PAGE
III	STUDIES RELATED TO THE MECHANISM OF ACTION OF MITOMYCIN B	78
	A. Introduction	78
	B. The Ethidium Bromide Fluorescence Assay	79
	C. Studies on the Covalent Interaction of Mitomycin B with DNA	84
	1. Detection of Covalent Cross-linking of DNA by Mitomycin B by the Ethidium Fluorescence Assay	84
	2. pKa of Mitomycin B	85
	3. Dependence of the Efficiency of Covalent Cross-Linking of DNA by Mitomycin B on the (G+C) Content of the DNA	85
	4. Cross-Linking by Mitomycin B Without Reduction	89
	5. Detection of Alkylation of DNA by Mitomycin B	89
	6. Correlation of the Decrease in Fluorescence With the Extent of Binding of Mitomycin B to DNA	93
	D. The Mechanism of DNA Degradation by Mitomycin B	96
	1. Detection of Mitomycin B Induced Single Strand Scission of CCC-DNA by the Fluorescence Assay	96
	2. Strand Scission of PM2 CCC-DNA by Covalently Bound Mitomycins	102
	3. Effect of Intercalated Ethidium on the Mitomycin B and Mitomycin C Induced Single Strand Scission and Cross-Linking of PM2 DNA	103
	E. Studies on Aziridine-Ring-Opened Mitomycin B	109

TABLE OF CONTENTS (continued)

CHAPTER			PAGE
III	F.	Electrochemical Studies of Mitomycin B and its Aziridine-Ring-Opened Derivative	110
	G.	Summary and Conclusions	114
	H.	Experimental	116
BIBLIOGRAPHY			128

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
I	Evidence for Interaction of Mitomycin C with DNA	5
II	Summary of Rate Constants for the Base-Catalyzed Solvolysis of 1-Methylgramine Methiodide 22 in Methanol	29
III	Activation Parameters for the Base-Catalyzed Methanolysis of 1-Methylgramine Methiodide 22	32
IV	Summary of Rate Constants for the Base-Catalyzed Solvolysis of 1-Methylisogramine Methiodide 23 in Methanol	39
V	Activation Parameters for the Base-Catalyzed Methanolysis of 1-Methylisogramine Methiodide 23	42
VI	Summary of Rate Constants for the Base-Catalyzed Solvolysis of 1-Methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in Methanol	47
VII	Activation Parameters for the Base-Catalyzed Methanolysis of 1-Methylindol-2-ylmethyl N-*t*-butylcarbamate 24	50
VIII	Dependence of Efficiency of Covalent Cross-Linking by Gramine Methiodide 26 on the (G+C) Content of the DNA	53
IX	Per Cent Cross-Linking of λ DNA by Mitomycin B *vs* pH	86
X	Average Molecular Weights of DNA's Determined by Sedimentation Velocities	88
XI	Dependence of Efficiency of Covalent Cross-Linking by Mitomycin B on the (G+C) Content of the DNA	90
XII	Per Cent Loss of Fluorescence in Alkylation of λ DNA by Mitomycin B *vs* pH	94
XIII	Correlation of Per Cent Loss of Fluorescence with the DNA-Mitomycin B Binding Ratio	97
XIV	Inhibition of Mitomycin B Induced Cleavage of PM2 CCC-DNA	123

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
1.	Effect of heat denaturation on uncross-linked, cross-linked, and sheared DNA	10
2.	Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 50.0°	22
3.	Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 60.0°	23
4.	Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 70.0°	24
5.	Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 80.0°	25
6.	Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 90.0°	26
7.	Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 with excess base in methanol at 80.0°	27
8.	Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 with excess 22 in methanol at 80.0°	28
9.	Plot of ln (k_1) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol	30
10.	Plot of ln (k_1/T) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol	31

LIST OF FIGURES (continued)

FIGURE	DESCRIPTION	PAGE
11.	Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol at 70.0°	35
12.	Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol at 80.0°	36
13.	Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol at 90.0°	37
14.	Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 with excess 23 in methanol at 90.0°	38
15.	Plot of ln (k_2) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol	40
16.	Plot of ln (k_2/T) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol	41
17.	Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol at 50.0°	44
18.	Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol at 70.0°	45
19.	Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol at 80.0°	46
20.	Plot of the ln (k_2) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol	48

LIST OF FIGURES (continued)

FIGURE	DESCRIPTION	PAGE
21.	Plot of ln (k_2/T) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol	49
22.	Dependence of efficiency of covalent cross-linking of DNA's by gramine methiodide 26 on the (G+C) content of the DNA	54
23.	Fluorescence assay for detecting covalent cross-linking of DNA	82
24.	Fluorescence assay for detecting single strand scission, cross-linking and alkylation of CCC-DNA	83
25.	The pH dependence of cross-linking of DNA by by reduced mitomycin B after 5 min	87
26.	Dependence of efficiency of covalent cross-linking of DNA's by reduced mitomycin B on the (G+C) content of the DNA	91
27.	The cross-linking of λ DNA by mitomycin B without reduction	92
28.	The pH dependence of alkylation of DNA by reduced mitomycin B	95
29.	Dependence of per cent loss of fluorescence on the DNA-mitomycin B binding ratio	98
30.	Single strand scission of PM2 CCC-DNA by mitomycin B	99
31.	Single strand scission of mitomycin B alkylated PM2 CCC-DNA	104
32.	Single strand scission of mitomycin C alkylated PM2 CCC-DNA	105
33.	Changes in fluorescence produced by mitomycin B in PM2 CCC-DNA treated with ethidium bromide	107
34.	Changes in fluorescence produced by mitomycin C in PM2 CCC-DNA treated with ethidium bromide	108

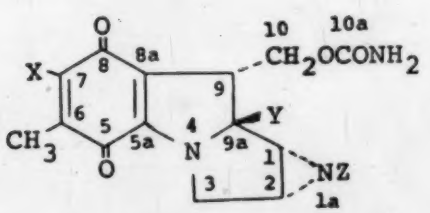
LIST OF FIGURES (continued)

FIGURE	DESCRIPTION	PAGE
35.	Single strand scission and alkylation of PM2 CCC-DNA by the mitomycin B aziridine-ring-opened derivative 55	111

CHAPTER I

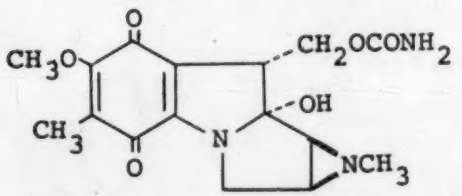
INTRODUCTION

The mitosane antibiotics 1 were first discovered in Japan in 1956 by Hata and co-workers[1], who isolated several members of the series from a culture of *Streptomyces caespitosus*. The basic ring structure was established by Webb *et al.*[2] This was confirmed by Tulinsky[3] who also determined the absolute configuration of mitomycin A. Mitomycin B 2 is unique among the mitomycins in that it has the opposite stereochemistry at three asymmetric centers.[4] The mitosanes contain three carcinostatic groups: quinone, urethane, and aziridine; and they were the first naturally occurring compounds found to contain the latter moiety.[5]



1

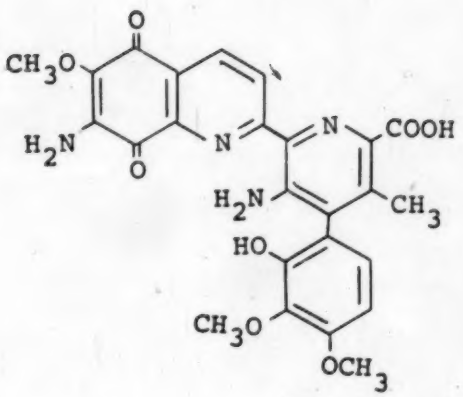
	X	Y	Z
Mitomycin A	OCH_3	OCH_3	H
N-Methylmitomycin A	OCH_3	OCH_3	CH_3
Mitomycin C	NH_2	OCH_3	H
Porfiromycin	NH_2	OCH_3	CH_3
7-Hydroxyporfiromycin	OH	OCH_3	CH_3



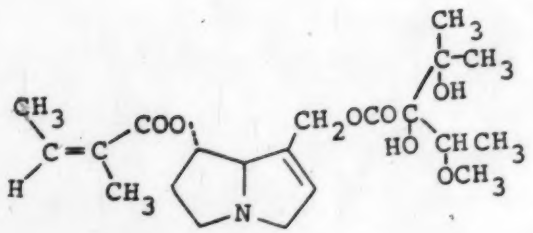
2

Further interest in the mitosanes is generated by the fact that they bear certain structural similarities to other antitumor agents, notably streptonigrin 3 and the pyrrolizidine alkaloids such as senkirkine, otosenine, and lasiocarpine 4.

Not only are the mitosanes effective against both gram positive and gram negative bacteria as well as certain viruses,[7] but they are also active against a number of types of tumor cells, including chronic myelogenous leukemia and solid tumors such as epithelial tumors, chorioepithelioma, reticulum cell sarcoma, and seminoma.[7,8] The two most active members of the series, mitomycin C and porfiromycin,



3



4

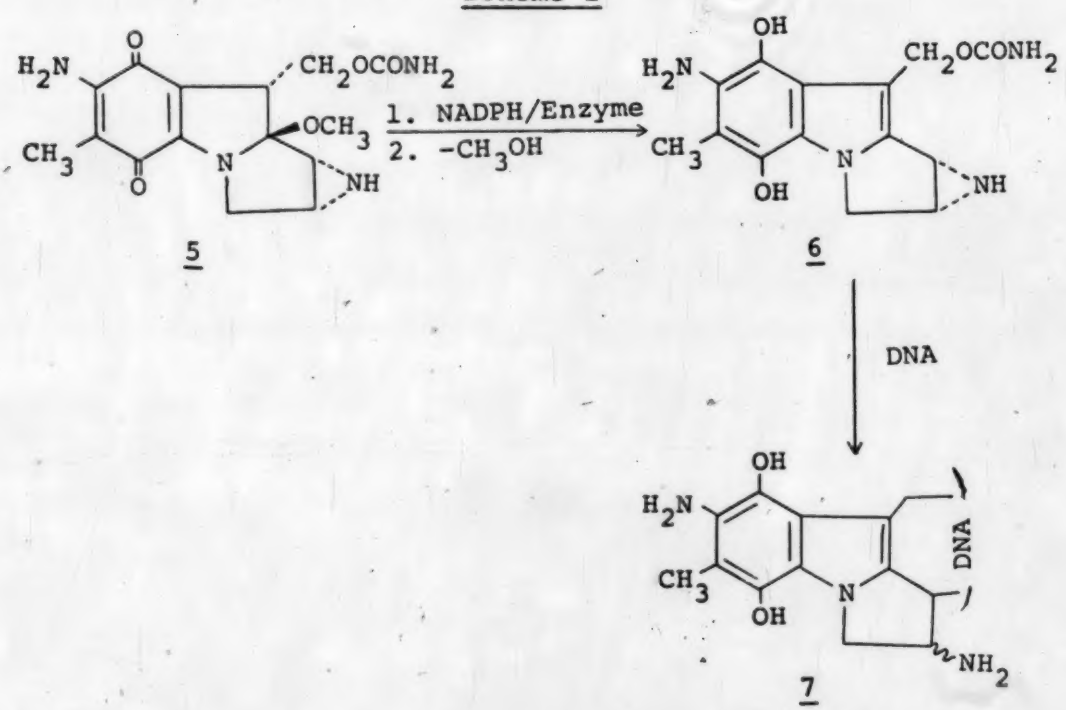
are now in clinical use in Japan for the treatment of a variety of carcinomas, especially those of the breast, stomach, lung, and colon.[9]

The mitosanes are toxic, although mitomycin B (MMB) has only about half the toxicity[10] of mitomycin C (MMC), which is sufficiently toxic that only 40 mg is used per course of treatment.

Little is known about mitomycin B specifically, though much of its mechanistic behavior may be expected to be analogous to that of other members of the mitosane family, particularly the extensively-studied mitomycin C. This compound 5 is believed to undergo an initial *in vivo* NADPH mediated reduction with a cellular reductase followed by the elimination of the elements of methanol to give 6[7] which is envisaged as cross-linking complementary strands of DNA in the manner shown in Scheme 1, although it has been sugges-

ted[11] that another binding site may be involved instead of the carbamate.

Scheme 1



5 6 7

Mitomycin C has also been shown[12] to cause DNA degradation through an oxidative process; evidence will be presented in Chapter III to show that this is also the case for mitomycin B. That DNA is the target of mitomycin C activity is indicated by the evidence presented in Table I.[13]

Cross-linking according to 7 requires the alkylation of DNA with the C-1 and C-10 positions of the mitosane system. This explanation is consistent with the observed reductive activation of the mitosanes to free the lone electron pair on the nitrogen atom at position 4 (8). This lone

Table I

Evidence for Interaction of Mitomycin C with DNA

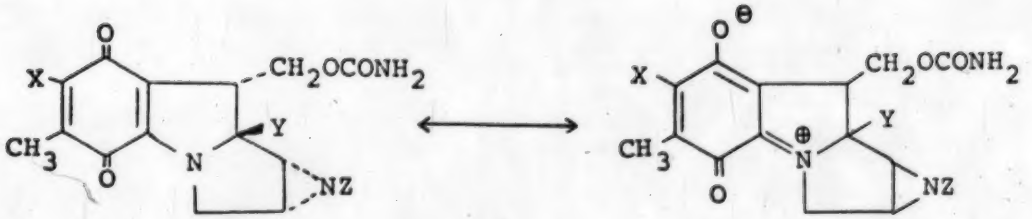
1. Bacteria exposed to mitomycin C exhibit an initial first order decline in viability that implies a single hit mechanism. This points to the bacterial chromosome as the site of action.[14]
2. Mitomycin C produces selective inhibition of monitored DNA synthesis in bacteria.[15]
3. Extensive degradation of DNA accompanies the administration of mitomycin C.[16]
4. Chromosome fragmentation is a result of DNA breakdown.[17]
5. Mitomycin C is mutagenic for both bacteria and *Drosophila*.[18]

---

pair can then assist in the elimination of the elements of HY (Scheme 2) and also serve to assist in the aziridine ring opening and carbamate expulsion while stabilizing cationic properties at positions 1 and 10 through resonance stabilization of structures 11 and 13[19b] (Scheme 3). Sartorelli[20] has suggested that the hydroquinone may also stabilize these sites (Scheme 4).

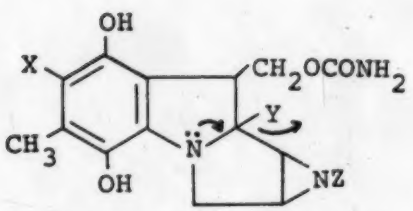
Cross-linking by the mitosanes was first established by Iyer and Szybalski[19] who found regeneration of double stranded DNA after heat denaturation and rapid

Scheme 2



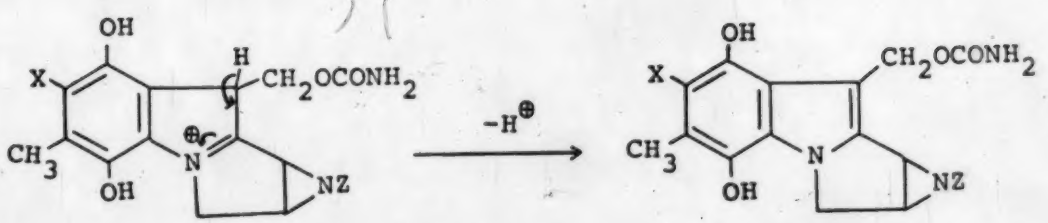
1

Reduction



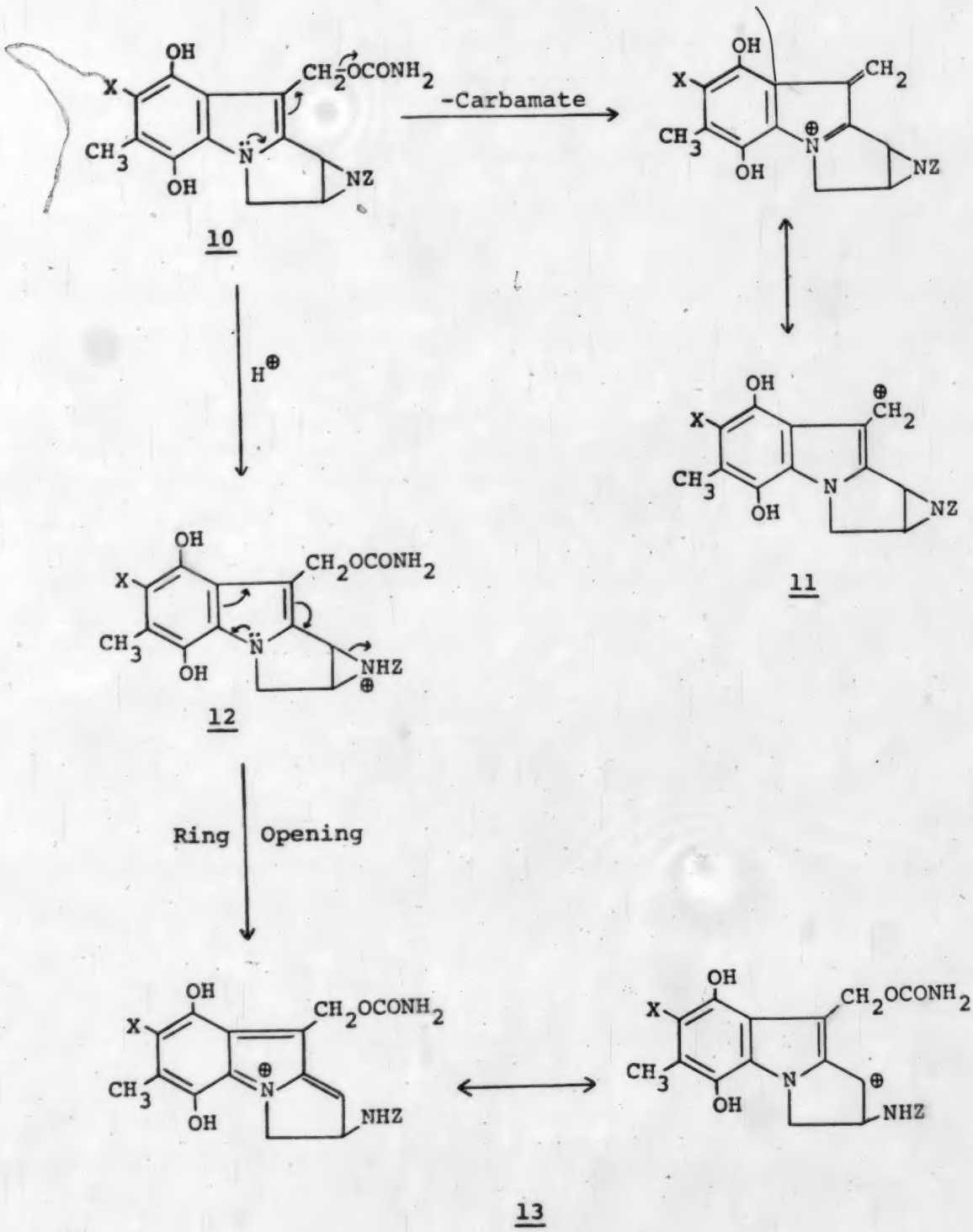
8

$-Y^{\oplus}$



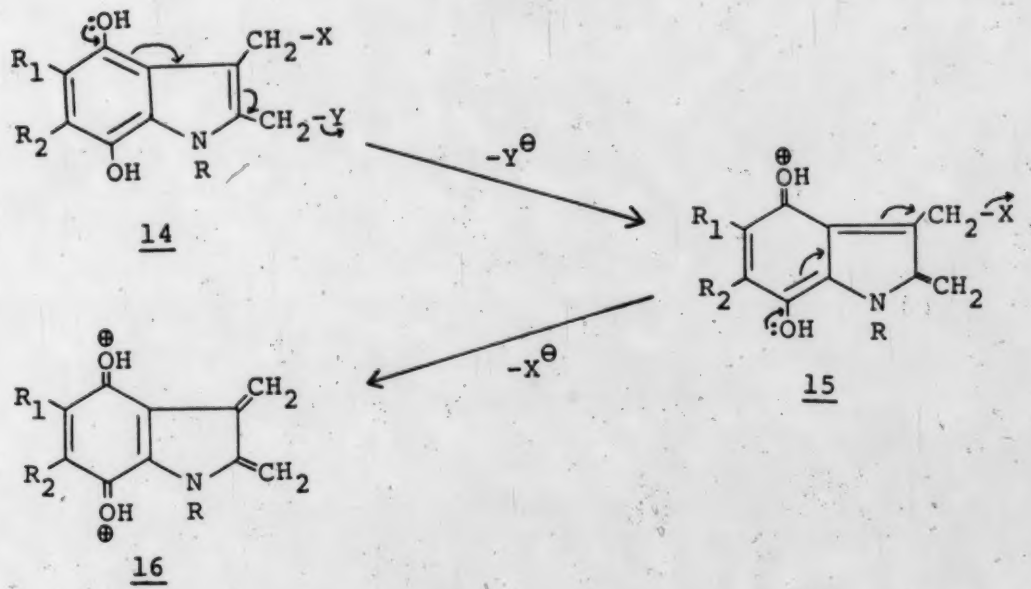
9 10

Scheme 3



10 11 12 13

Scheme 4



cooling of isolated bacterial DNA which had been treated with mitomycin C. Uncross-linked DNA will not renature after the heating/cooling process because the re-registering of the separated complementary strands is a slow, temperature-dependent process. However in covalently cross-linked DNA, the cross-link prevents the complete separation of the strands and serves as a nucleation site for the rapid renaturation of the double strand. It has been found that this renaturation will proceed at a rate of 10^7 - 10^8 base pairs per second once a nucleation point has been established.[21] Such cross-links of the DNA are infrequent as has been shown by shearing experiments which produced a decrease in the amount of regeneration after heat denaturation of double stranded DNA. Estimates

have placed the frequency of cross-linking as low as one cross-link per 20,000 base pairs[19] and not exceeding one per 1,000 base pairs.[20] The behavior of uncross-linked, cross-linked, and sheared DNA is illustrated in Figure 1.

The relative rarity of cross-links has also hindered study of the sites on both the mitomycins and the DNA which are involved in the cross-linking process. It is known that exchanging the 7-amino and 7-methoxy groups affects the cross-linking ability only slightly, although the nature of this substituent may alter the redox potential of the quinone and hence also the biological ability.[22] Replacement of the methoxy group in the 9a position with an hydroxy group diminishes the activity of the mitosane, while substitution with hydrogen renders the compound inactive. Yet the derivative of mitomycin B in which the elements of water have been eliminated from the 9-9a positions (17) is orally active as an antibacterial agent,[23] suggesting that the 9a position is not involved in the alkylation process. Mitomycins of structure 1 are active if Z = hydrogen or alkyl, but show little or no activity if Z = acyl; nor are aziridine ring-opened derivatives of 1 found to cross-link. Replacement of the carbamate moiety by hydroxyl renders the derivative less active, while replacement by methoxyl suppresses all activity. Thus there is evidence implicating both the aziridine ring and the carbamate function in the process of alkylation. Recently Tomasz *et al.*[24]

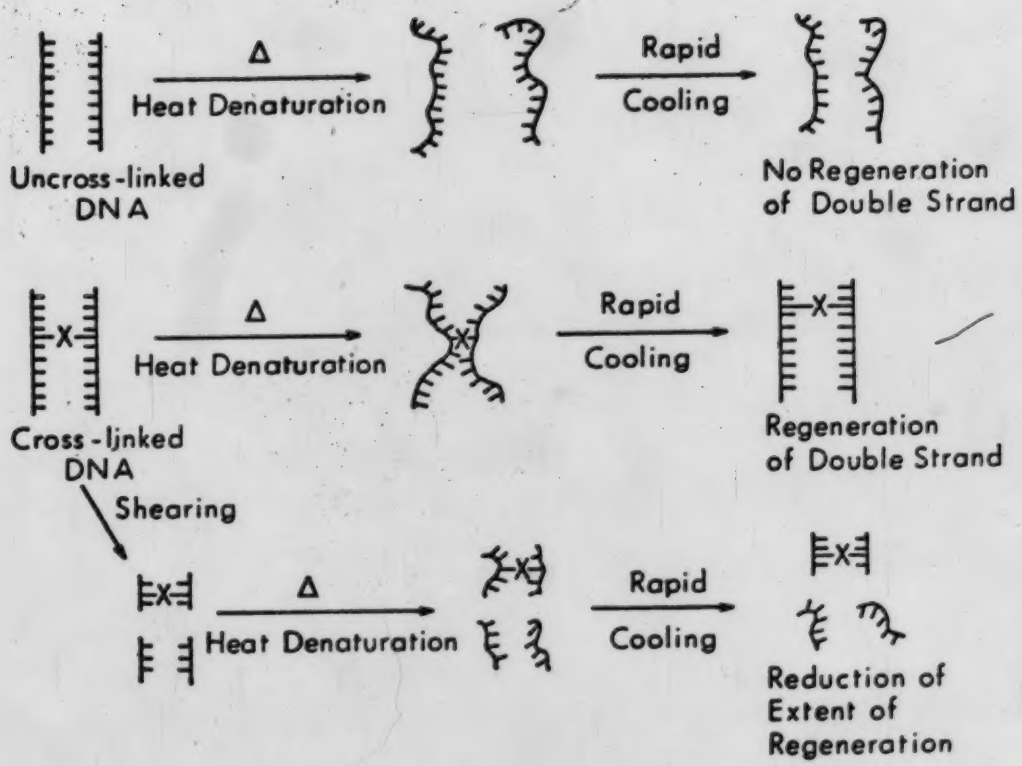
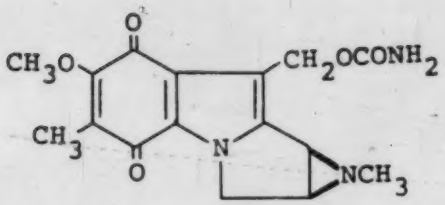


Figure 1. Effect of heat denaturation on uncross-linked, cross-linked, and sheared DNA.



17

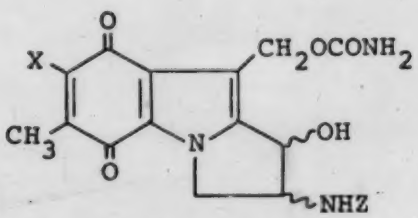
have suggested that the semiquinone form of mitomycin C may be significantly involved in the covalent binding of DNA.

Iyer and Szybalski[19b] have shown that the extent of cross-linking varies as the (G+C) content of the DNA. Coupled with this is the study of the alkylation of nucleoside homopolymers with ^{14}C-labelled porfiromycin in which it was found that guanosine polymers were alkylated at least four times as extensively as any of the other polymers.[25] It would therefore appear that the guanine residues of the DNA are the most likely sites for alkylation.

Nitrogen and sulfur mustards have been postulated to cross-link between the N-7 positions of guanines on opposite DNA strands.[26] Yet the examination of space-filling models shows that the 4.3 Å span between positions C-1 and C-10 of the mitomycins 1, or the 2.8 Å span of the activated forms (10), can best be accommodated by

links between two O-6 positions of guanines.[19c]

Alkylation of DNA as a process distinct from cross-linking has been studied by Weissbach and Lisio[27] who found attachment of mitomycin C and porfiromycin as high as one molecule per 1,000 base pairs and found that only approximately 10% of the mitomycin molecules which alkylate DNA will actually cross-link. This process may well account for the residual biological activity observed for aziridine ring-opened species such as 18.[22b,c] A recent assay developed by Tomasz[28] mitigates against the involvement of the guanine N-7 position as the site of alkylation.



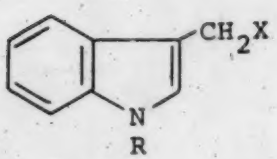
18

Mitomycin C has also been found to cause extensive degradation of DNA,[29] although this is much slower than the cross-linking process.

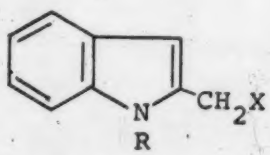
Since little or no information was available on the mode of action of mitomycin B, the cross-linking, alkylation, and degradation processes induced by this

compound are studied and compared in Chapter III with those previously reported[13] for mitomycin C.

Other than the work of Albright and Snyder,[30,31] little is known about the reactivity of even simply substituted indolylcarbinyl systems of the types 19 and 20,



19



20

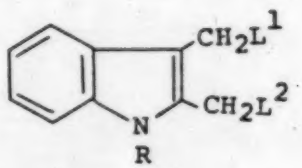
which are structurally analogous to the activated mitomycins.

In Chapter II a kinetic study of the base-catalyzed methanolysis of some of these systems is undertaken in order to gain an insight into the relative reactivity of the two potential alkylating sites, as well as the comparative reactivity of the trialkylammonium and carbamate leaving groups. Both of these objectives are achieved through a study of 19 in which R = H or R = CH_3 and X = $^{\oplus}N(CH_3)_3\ I^{\ominus}$ and 20 in which R = CH_3 and X = $^{\oplus}N(CH_3)_3\ I^{\ominus}$ or X = $OCONHC(CH_3)_3$. Interaction of these compounds with DNA is also investigated.

CHAPTER II

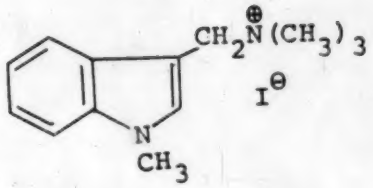
KINETIC STUDY OF 2- AND 3-INDOLYLCARBINYL COMPOUNDS

The reduced form of the mitomycins (10) is structurally related to the disubstituted dicarbinylindole 21. In order to gain a deeper insight into the mode of

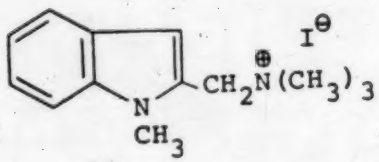


21

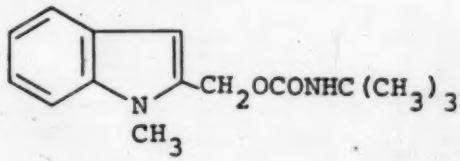
action of the mitomycins and the step-wise nature of the cross-linking process, the carbinyl reaction sites were studied separately on model compounds with the same leaving group. Also, one of the reacting sites was studied with different leaving groups, analogous to those present in the mitomycins. Thus a comparison of 1-methylgramine methiodide 22 and 1-methylisogramine methiodide 23 afforded information on the relative reactivities of the two sites, while a comparison of 23 and 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 allowed a quantitative comparison of the reactivities of the respective leaving groups. Unfortunately, it was not possible to synthesize



22

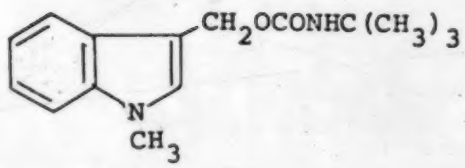


23

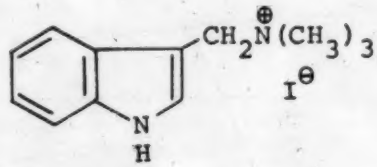


24

the isomeric carbamate 25 to serve as a cross-check, though this failure in itself is consistent with the other findings for this series of compounds. Gramine methiodide 26 was also investigated in conjunction with the study of its N-methyl derivative 22.



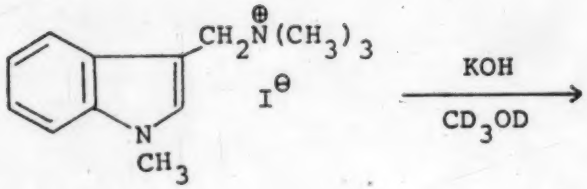
25



26

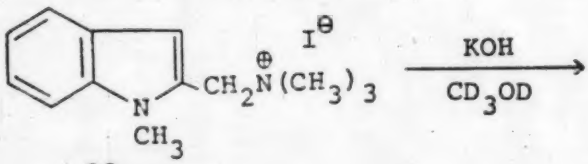
The reaction studied for these compounds was the hydroxide-catalyzed solvolysis in methanol. By performing the reaction with the gramine derivatives (Scheme 5)

Scheme 5



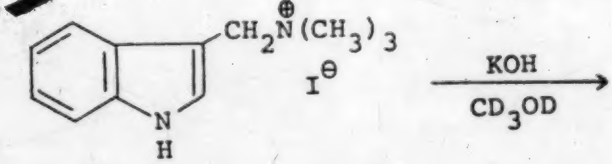
22 27

KI + HOD + $N(CH_3)_3$



23 28

KI + HOD + $N(CH_3)_3$



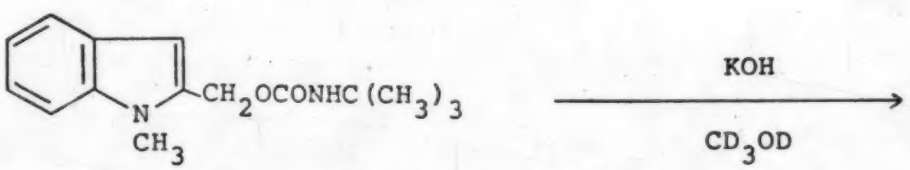
26 29

KI + HOD + $N(CH_3)_3$

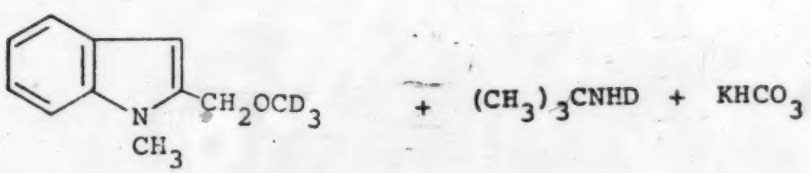
in a sealed nmr tube, using deuterated methanol, it was possible to follow kinetically the disappearance of the singlet in the nmr spectrum due to $-\overset{\oplus}{N}(CH_3)_3$ (3.09 δ from hexamethyldisilane (HMDS) for 22, 3.13 δ from HMDS for 23) together with the appearance of the singlet at 2.18 δ from HMDS due to $N(CH_3)_3$.

The corresponding reaction of the carbamate 24 (Scheme 6) gave the same indole product. However, the nmr absorption of the *t*-butyl group in the product had virtually the same chemical shift as that of the *t*-butyl group in 24; and so this signal could not be followed in

Scheme 6



24



28

a kinetic study. However, the methylene protons absorbed at 5.12 δ from HMDS in the carbamate 24 and at 4.68 δ from HMDS in the ether 28, thus facilitating a kinetic study.

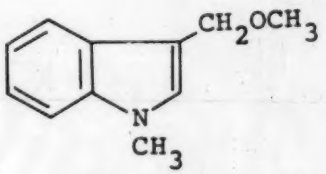
1-Methylgramine Methiodide 22

Synthesis

This compound was synthesized as shown in Scheme 7.

Product Runs

The product 36 of the solvolysis reaction was established by carrying the reaction to completion in meth-

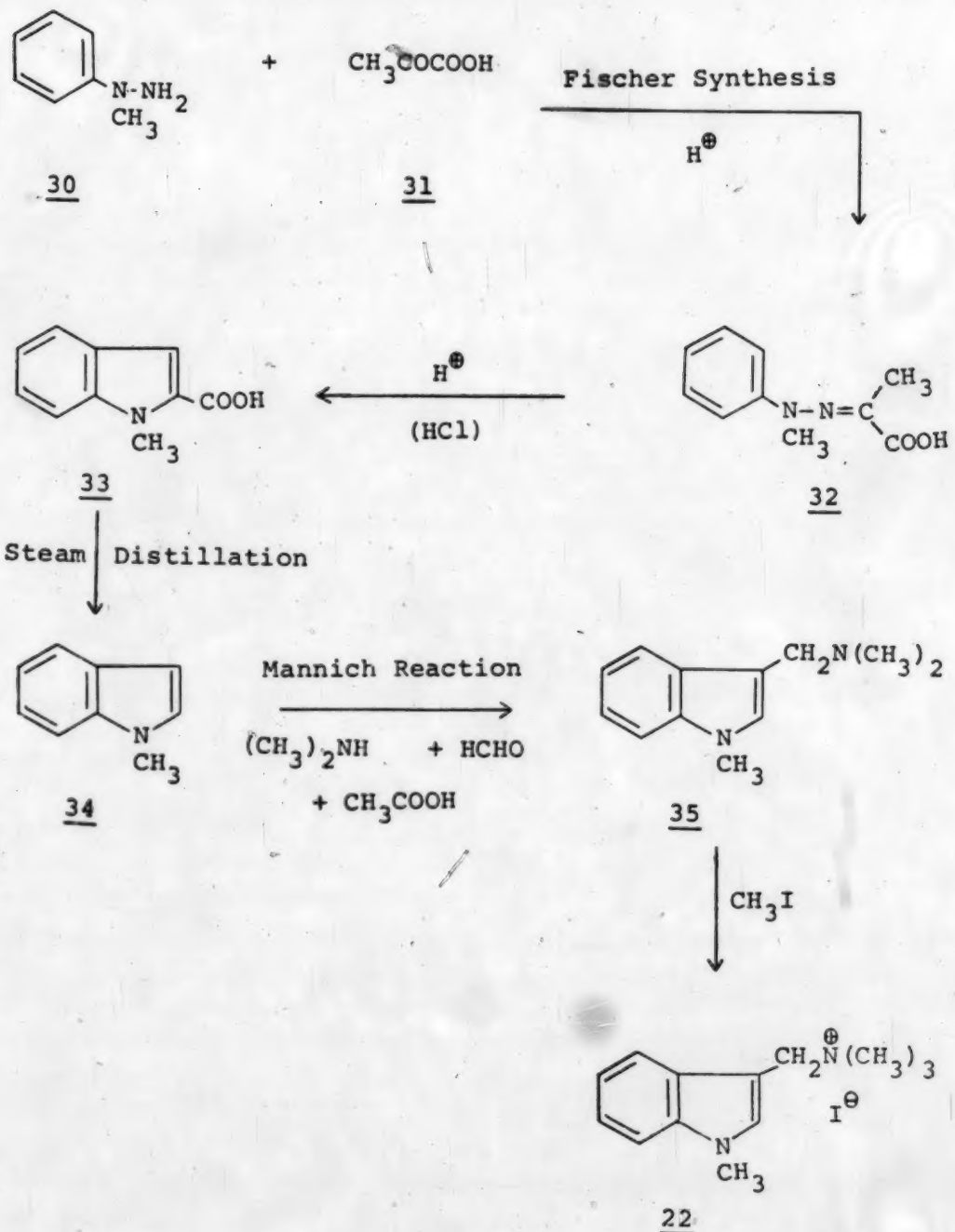


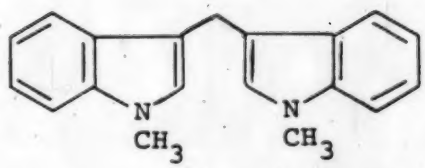
36

anol with potassium hydroxide, and isolation of the product as a brown oil. Recovery was quantitative and the structure of the product was deduced by assignment of the nmr spectrum.

A similar reaction in the absence of base yielded only an intractable brown material. A reaction performed with sodium hydroxide as base and water as solvent qave an orange oil. The mass spectrum (m = 274) and the nmr spectrum are consistent with the structure 37, which is in accord with the known chemistry of hydroxymethylindoles (such as the presumed intermediate) in the presence of base.[32]

Scheme 7

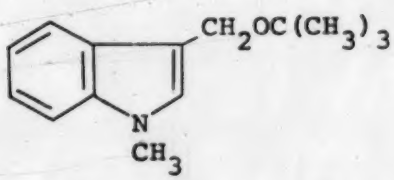

N-NH2
CH3
+
CH3COCOOH
Fischer Synthesis
H⊕
30
31
N-N=C
CH3
COOH
32
H⊕
(HCl)
N
CH3
COOH
33
Steam Distillation
N
CH3
34
Mannich Reaction
(CH3)2NH + HCHO
+ CH3COOH
CH2N(CH3)2
35
CH3I
CH2N⊕(CH3)3
I⊖
22



37

Use of the weaker, non-nucleophilic base, 2,6-lutidine, gave a small amount of material identifiable as the ether 36, but also a number of other unidentified products.

When a suspension of sodium methoxide in *t*-butyl alcohol was used, 22 gave a small amount of yellowish oil which by nmr and mass spectra was identified as the *t*-butyl ether 38.



38

Kinetic Study

The first reaction of Scheme 5 was studied at five temperatures: 50.0, 60.0, 70.0, 80.0, and 90.0°. In all cases a plot of the integrated first-order rate expression against time gave a straight line. Two additional kinetic runs were carried out at 80.0°, in which the base concentration was in turn approximately doubled and halved. The reaction with 0.428 equivalent of base proceeded at the same rate (within experimental error) as the original run until the base was nearly consumed, then slowed suddenly. The run with 2.09 equivalents of base proceeded at a rate slightly faster than the original; however the increase was not sufficient to justify supposing a higher order of reaction, and may be the result of changing medium effects.

The results of the kinetic measurements are summarized in Table II, with the rate constants being calculated from the slopes of the plots shown in Figures 2 to 8.

Using these rate constants to plot ln (k_1) *vs* (1/T) and ln (k_1/T) *vs* (1/T) (Figures 9 and 10 respectively), the activation parameters Ea, $\Delta H^{\neq}$, and $\Delta S^{\neq}$ were calculated.

From Figure 9, Ea was obtained as -R x Slope, and $\Delta S^{\neq}$ was obtained as R x (Intercept - 23.767).[33] The results are tabulated in Table III.

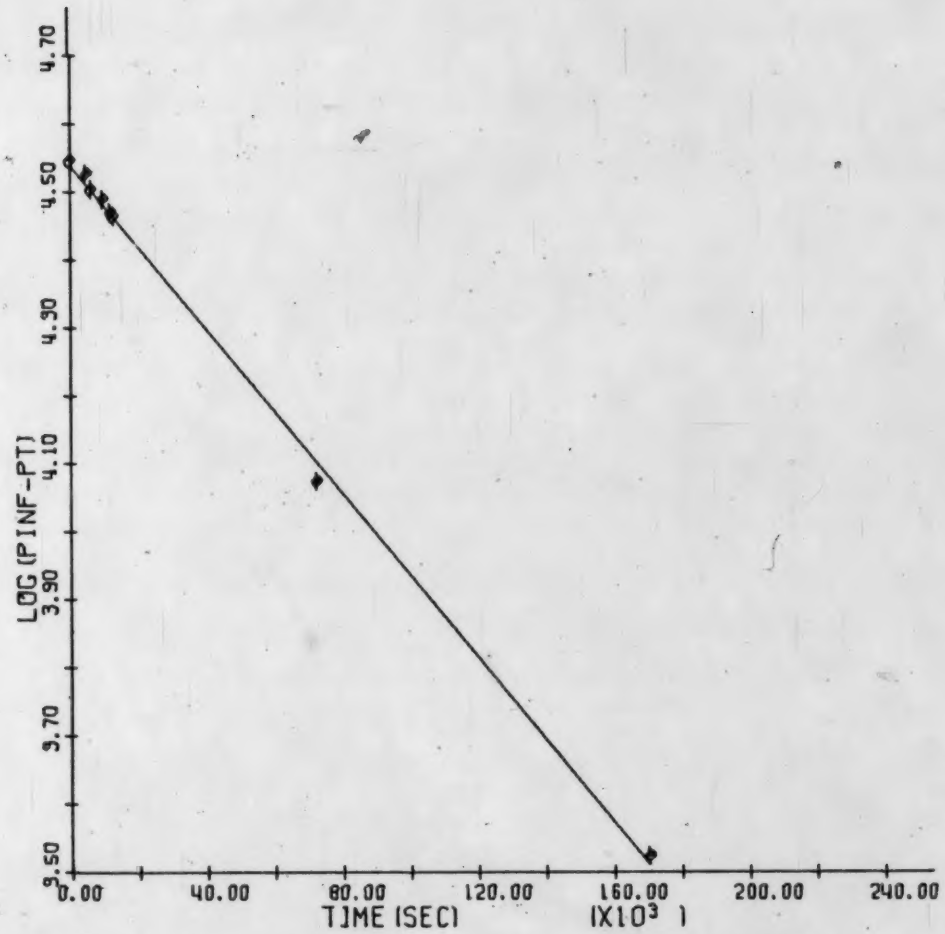


Figure 2. Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 50.0°. PINF = per cent reaction at infinite time; PT = per cent reaction at time T; logarithms are to base e; k_1 = -Slope.

Note: The expression 10^n appearing on the axes of Figures 2 to 22 and 26, and in the headings of Tables VIII, IX, XI, XII, and XIII, indicates that the quantity to which it applies must be multiplied by that factor.

mark on original

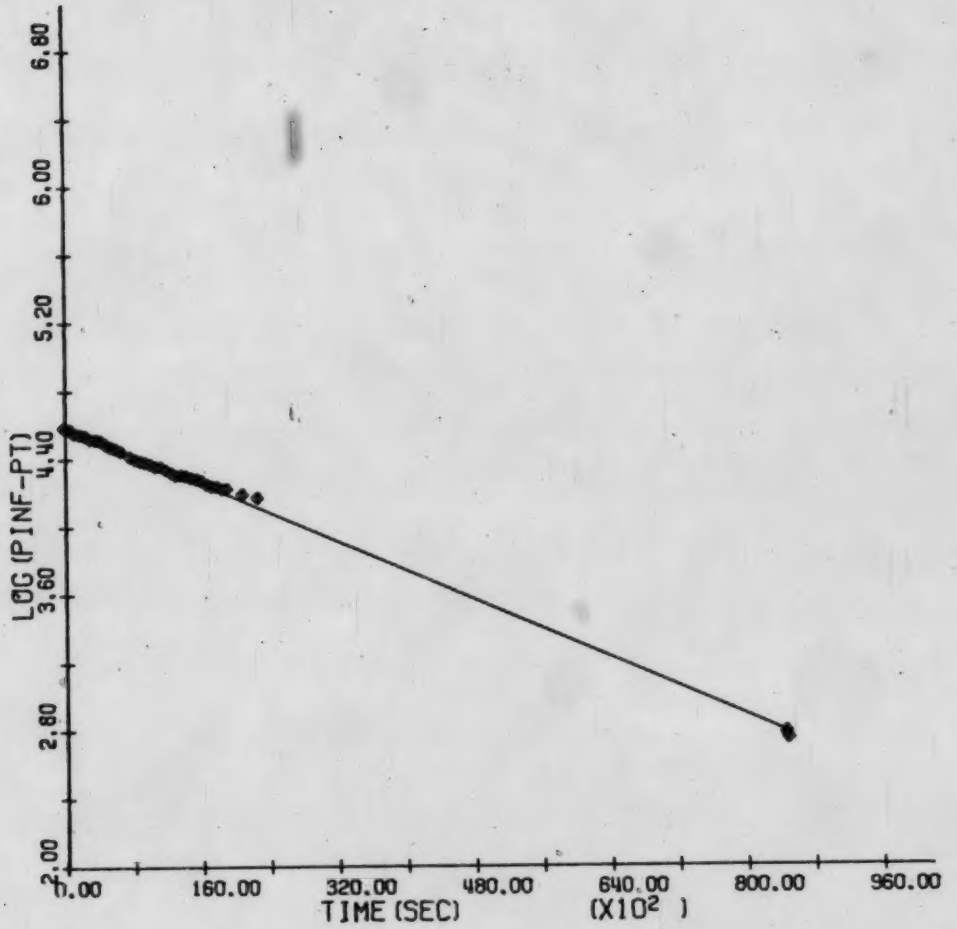


Figure 3. Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 60.0°. Legend as for Figure 2.

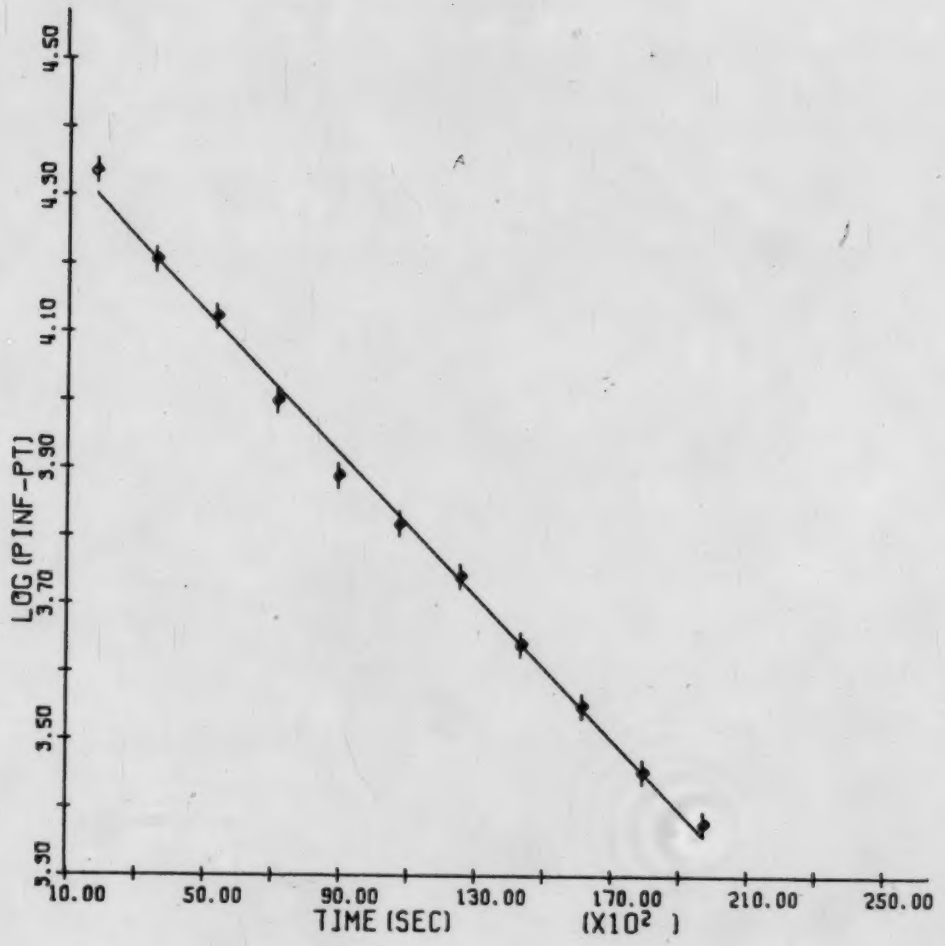


Figure 4. Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 70.0°. Legend as for Figure 2.

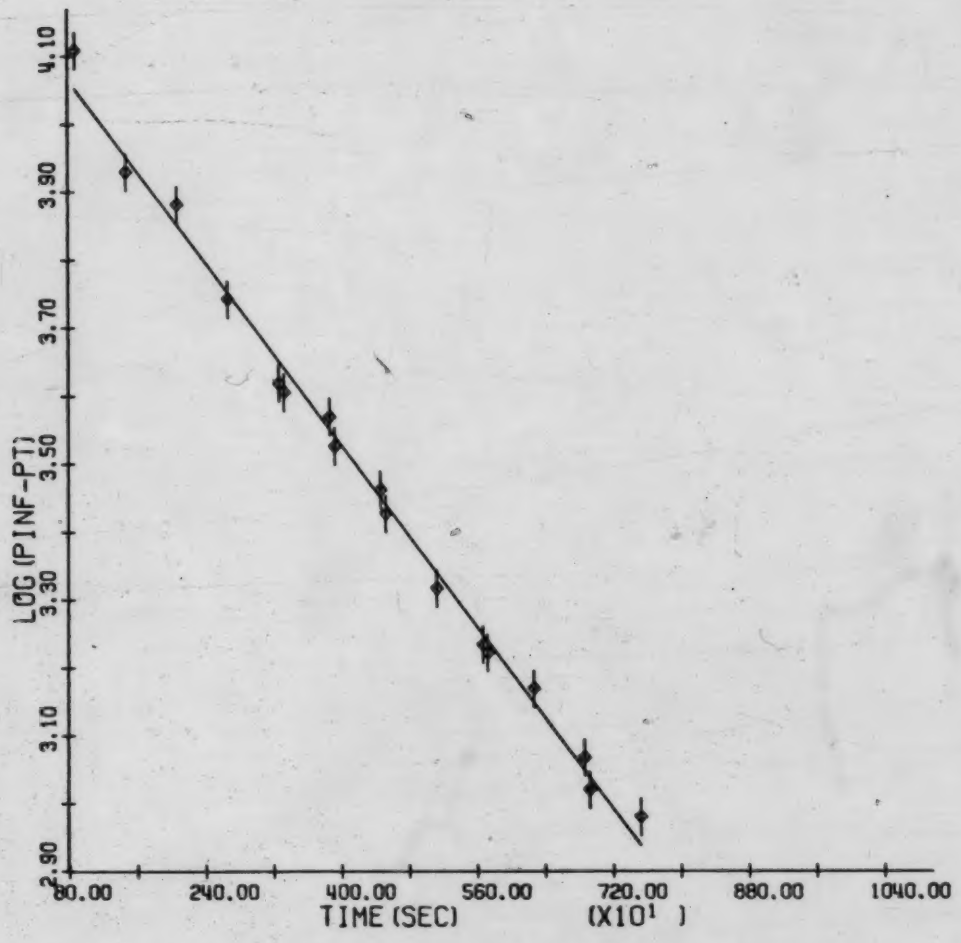


Figure 5. Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 80.0°. Legend as for Figure 2.

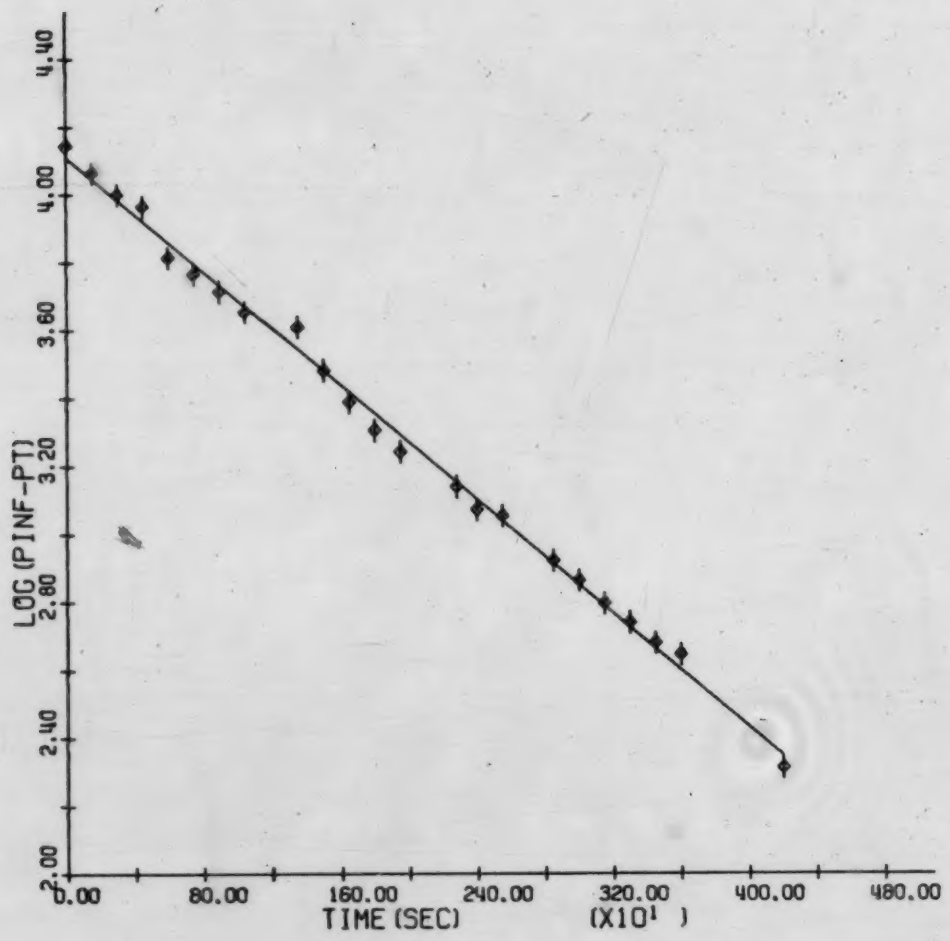


Figure 6. Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol at 90.0°. Legend as for Figure 2.

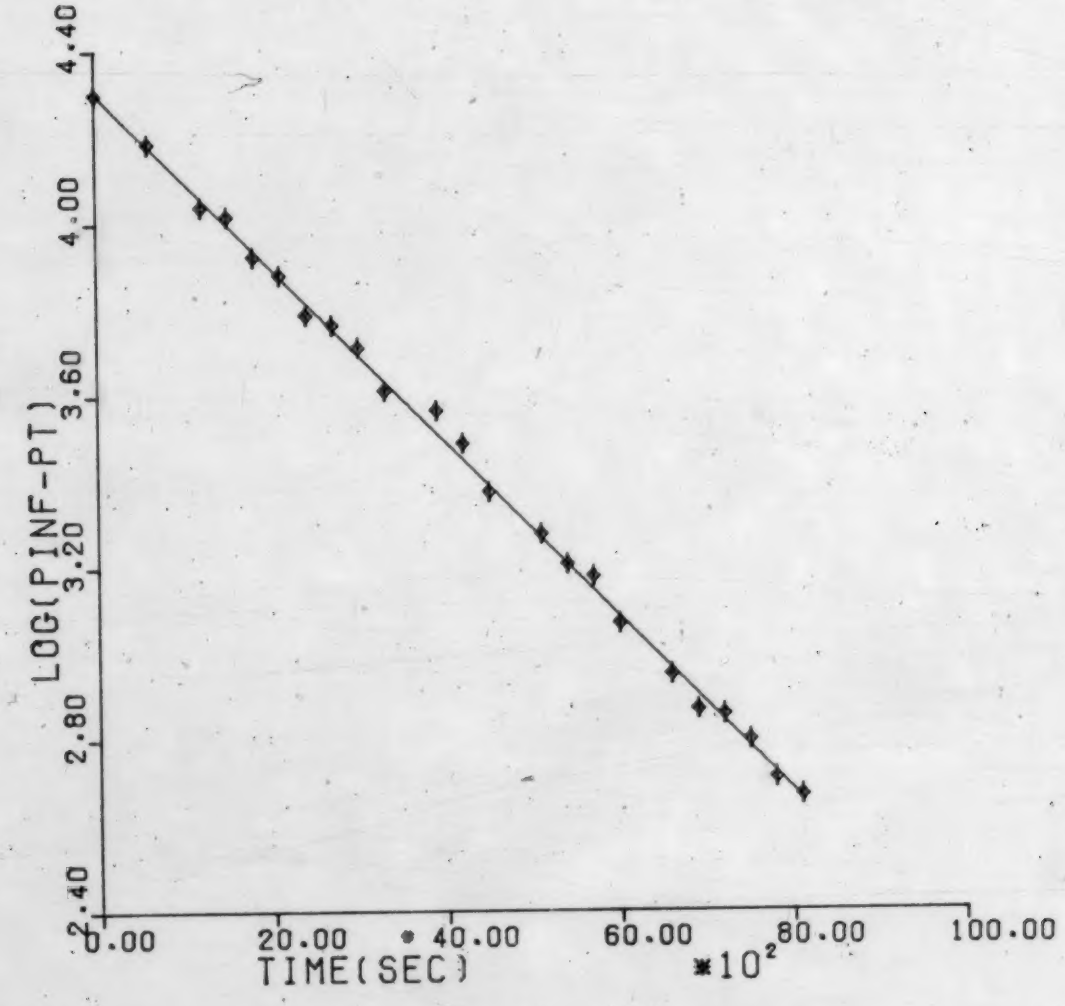


Figure 7. Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 with excess base in methanol at 80.0°. Legend as for Figure 2.

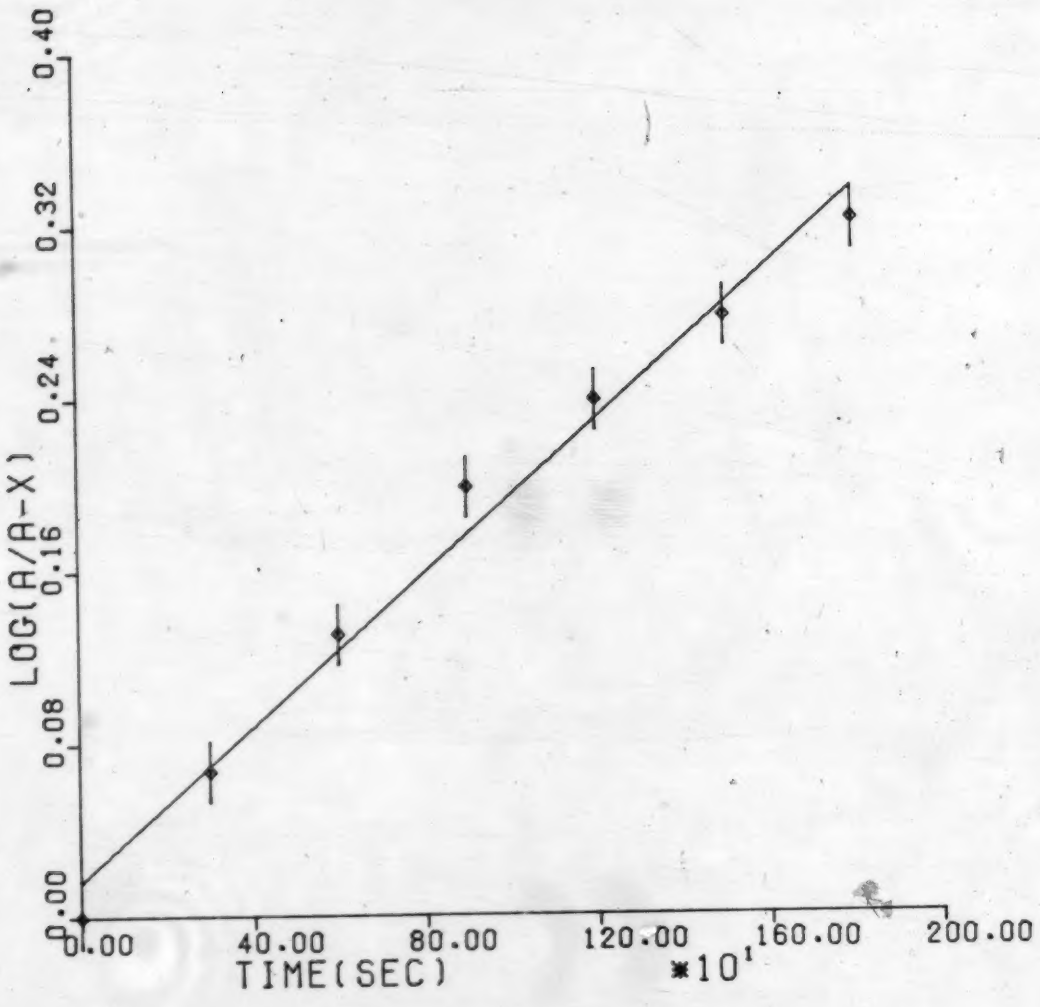


Figure 8. Plot of the integrated first-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 with excess 22 in methanol at 80.0°. A = initial concentration of substrate; X = concentration of substrate reacted at time T; logarithms are to base e; k_1 = Slope.

Table II

Summary of Rate Constants for the Base-Catalyzed Solvolysis of 1-Methylgramine Methiodide 22 in Methanol

Run	[KOH]	[22]	[KOH]:[22]	Temp (°C)	k_1 (sec^{-1})	Figure
II-115	0.0977	0.0978	0.990	50.0	$(6.04 \pm 0.09) \times 10^{-6}$	2
II-155	0.1411	0.1016	1.389	60.0	$(2.14 \pm 0.01) \times 10^{-5}$	3
II-123	0.1105	0.1153	0.958	70.0	$(5.24 \pm 0.01) \times 10^{-5}$	4
II-147	0.1075	0.1078	0.997	80.0	$(1.67 \pm 0.04) \times 10^{-4}$	5
II-159	0.1184	0.1083	1.093	90.0	$(4.20 \pm 0.06) \times 10^{-4}$	6
III-73	0.2228	0.1069	2.084	80.0	$(2.03 \pm 0.02) \times 10^{-4}$	7
III-75	0.0452	0.1060	0.426	80.0	$(1.78 \pm 0.10) \times 10^{-4}$	8

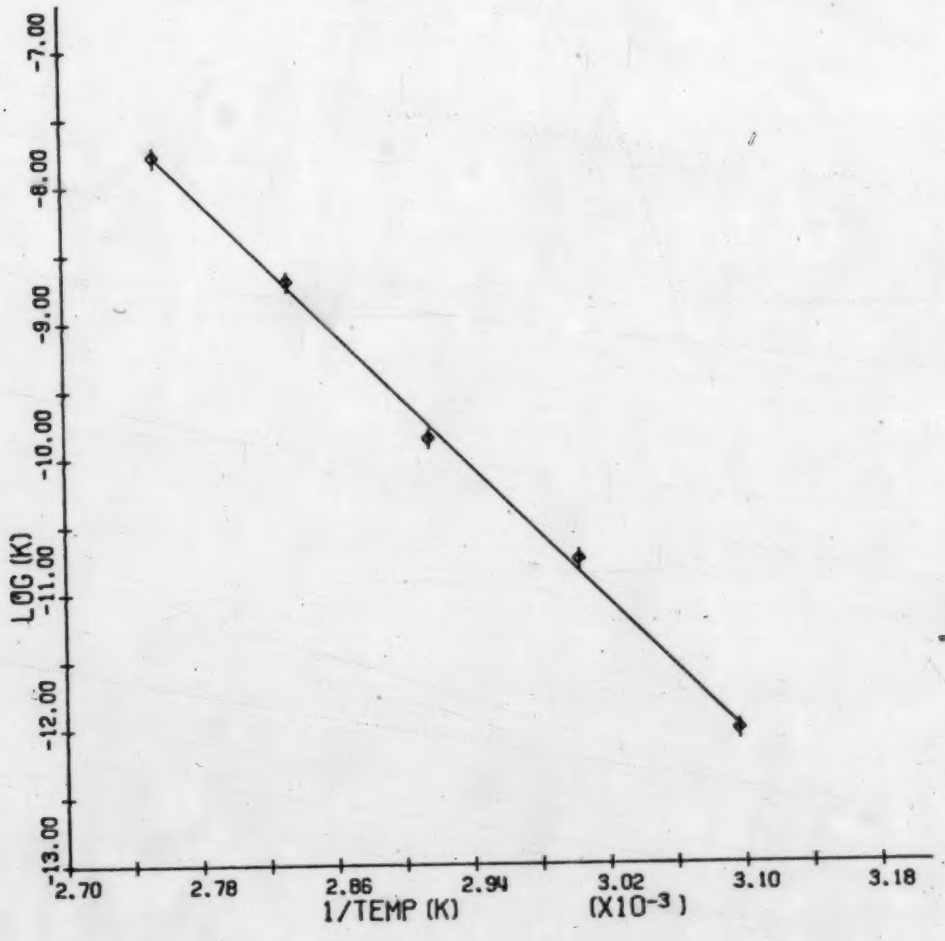


Figure 9. Plot of ln (k_1) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol. k = rate constant; TEMP(K) = absolute temperature; logarithms are to base e.

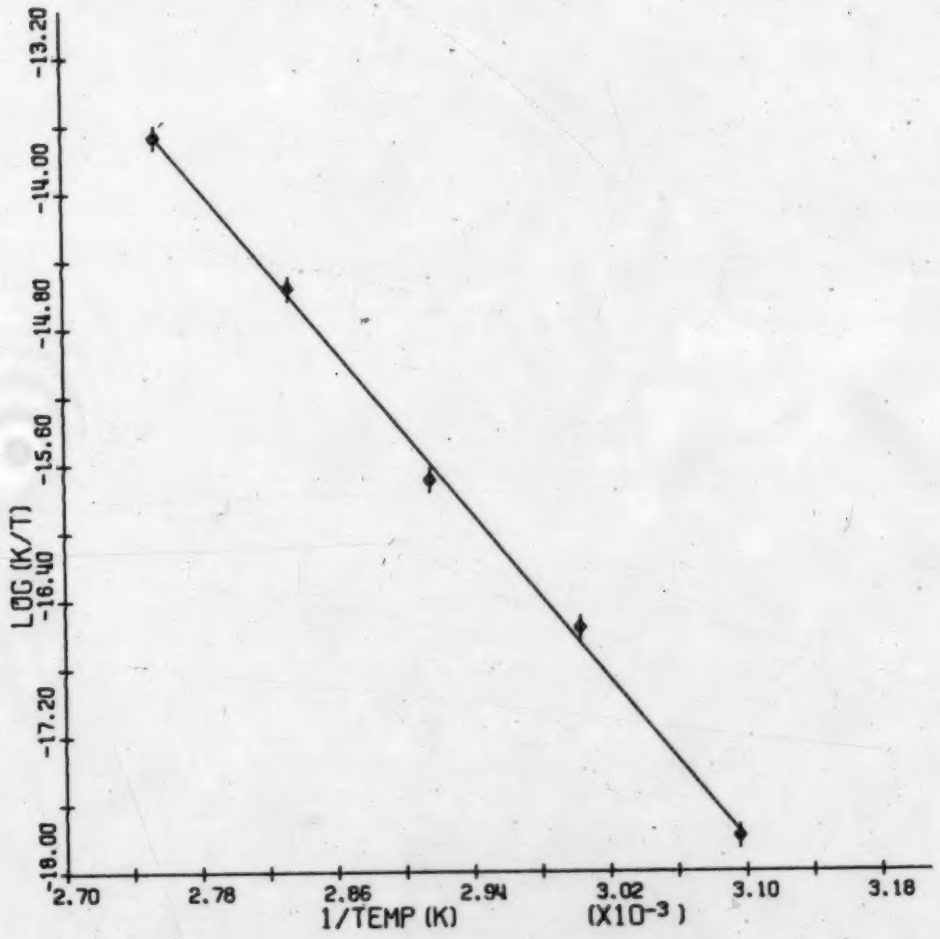


Figure 10. Plot of ln (k_1/T) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylgramine methiodide 22 in methanol. k = rate constant; T = TEMP(K) = absolute temperature; logarithms are to base e.

Table III

Activation Parameters for the Base-Catalyzed Methanolysis of 1-Methylgramine Methiodide 22

Ea	24.6 ± 0.6 kcal/mole
$\Delta H^{\neq}$	23.8 ± 0.6 kcal/mole
$\Delta S^{\neq}$	-8.5 ± 1.8 e.u.

DNA Study

By the fluorescence assay described in Chapter III there was no indication that 22 alkylates PM2 DNA at pH 7.0 and 37°.

1-Methylisogramine Methiodide 23

Synthesis

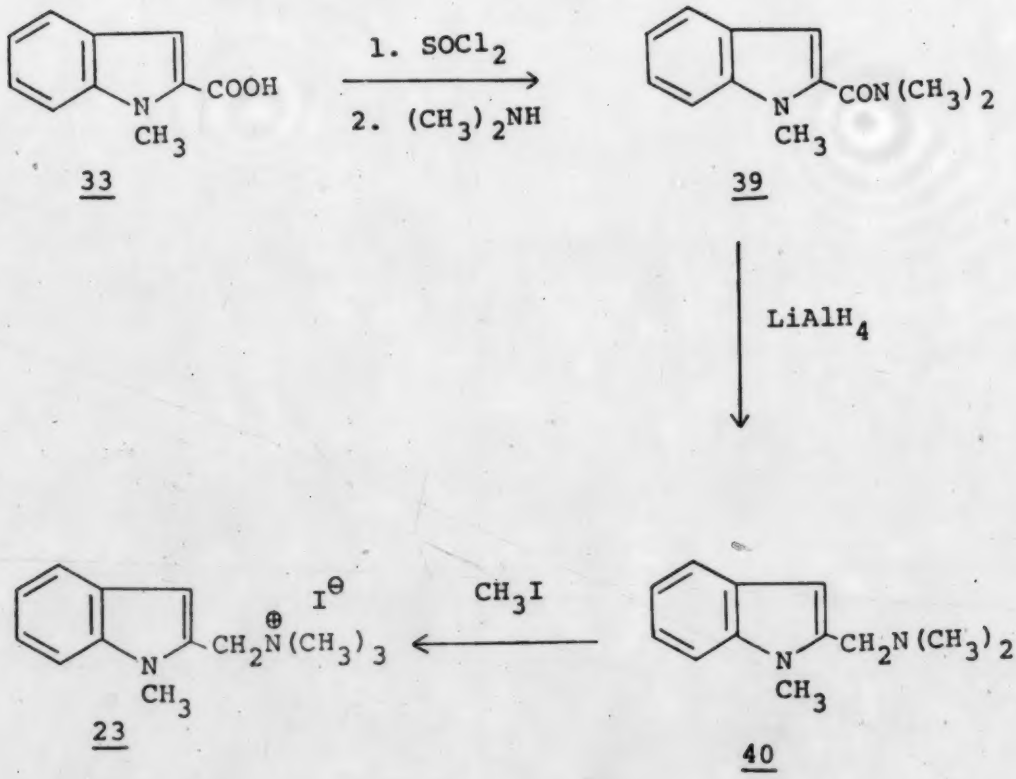
This compound was synthesized as shown in Scheme 8.

Product Runs

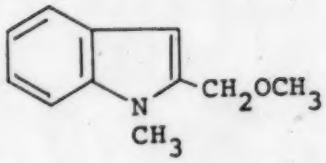
The product 41 of the solvolysis reaction was established by carrying the reaction to partial completion and isolating 31% of product 41 as a yellow solid, which was identified by its nmr spectrum; 69% of the product was unreacted 23. The total mole recovery of material (based on the indole moiety) was 94%.

A similar run using sodium hydroxide as base and water as solvent gave only recovered starting material.

Scheme 8



33 39 40 23



41

Kinetic Study

The second reaction of Scheme 5 was studied at three temperatures: 70.0, 80.0, and 90.0°. This reaction was found to follow second-order kinetics, as determined from a plot of the integrated second-order rate expression against time. An additional run at 90.0° with 0.428 of the original amount of base proceeded at a rate consistent with this mechanism; the increase would not justify a different order and again may be the result of changing medium effects.

The results of the kinetic measurements, based on the plots shown in Figures 11 to 14, are given in Table IV.

From the observed rate constants the activation parameters, based on plots of ln (k_2) *vs* (1/T) (Figure 15) and ln (k_2/T) *vs* (1/T) (Figure 16), were calculated as for 1-methylgramine methiodide 22. These are tabulated in

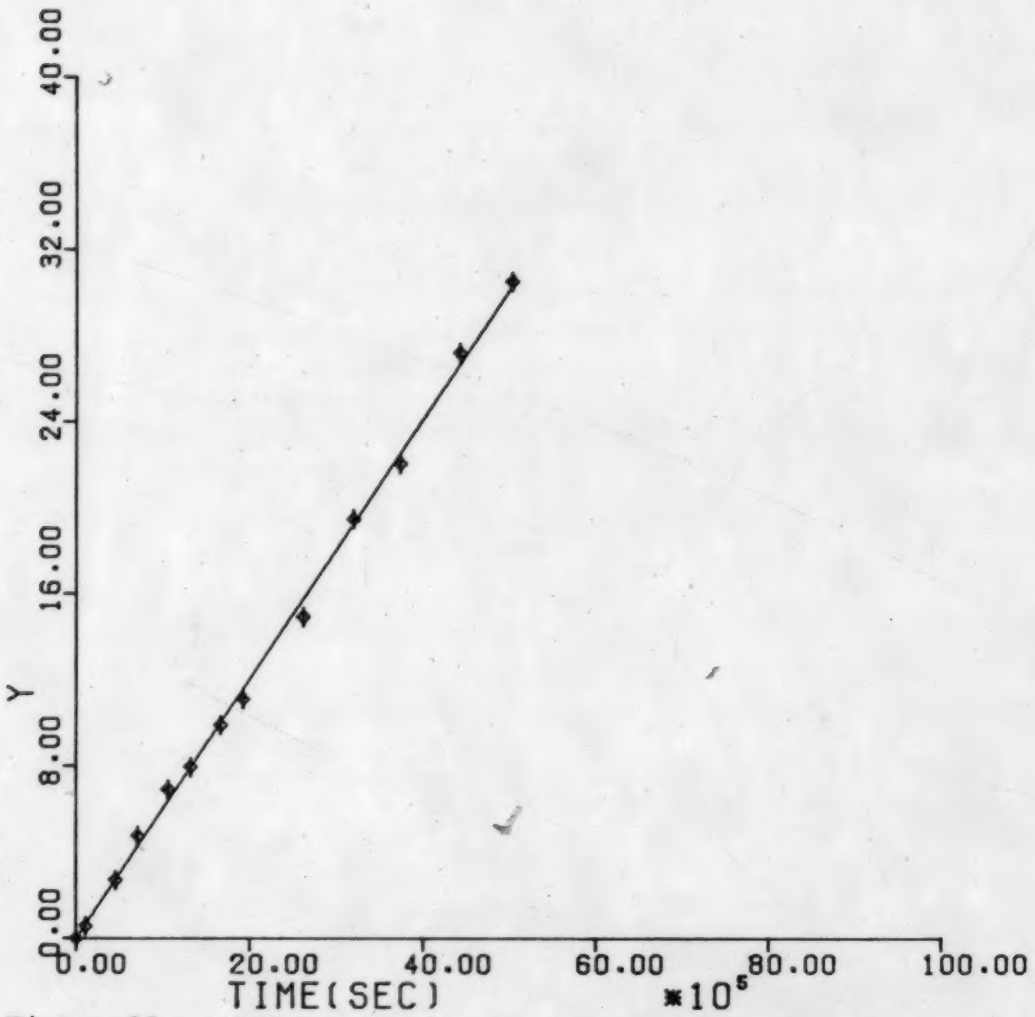


Figure 11. Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol at 70.0°.

$Y = (1/(b_o-a_o))\ln(a_o(b_o-x)/b_o(a_o-x))$ where a_o is the initial concentration of substrate, b_o is the initial concentration of base, and x is the concentration of substrate reacted at time T; k_2 = Slope.

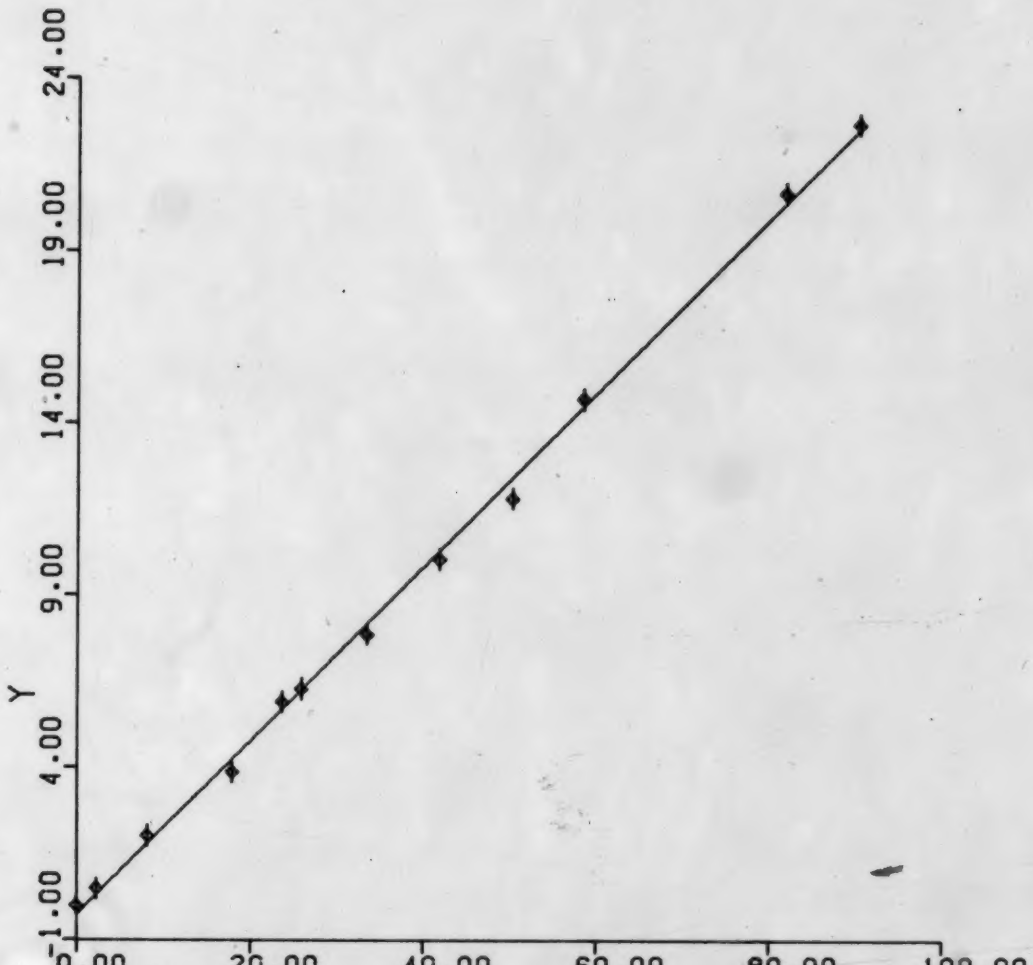


Figure 12. Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol at 80.0°. Legend as for Figure 11.

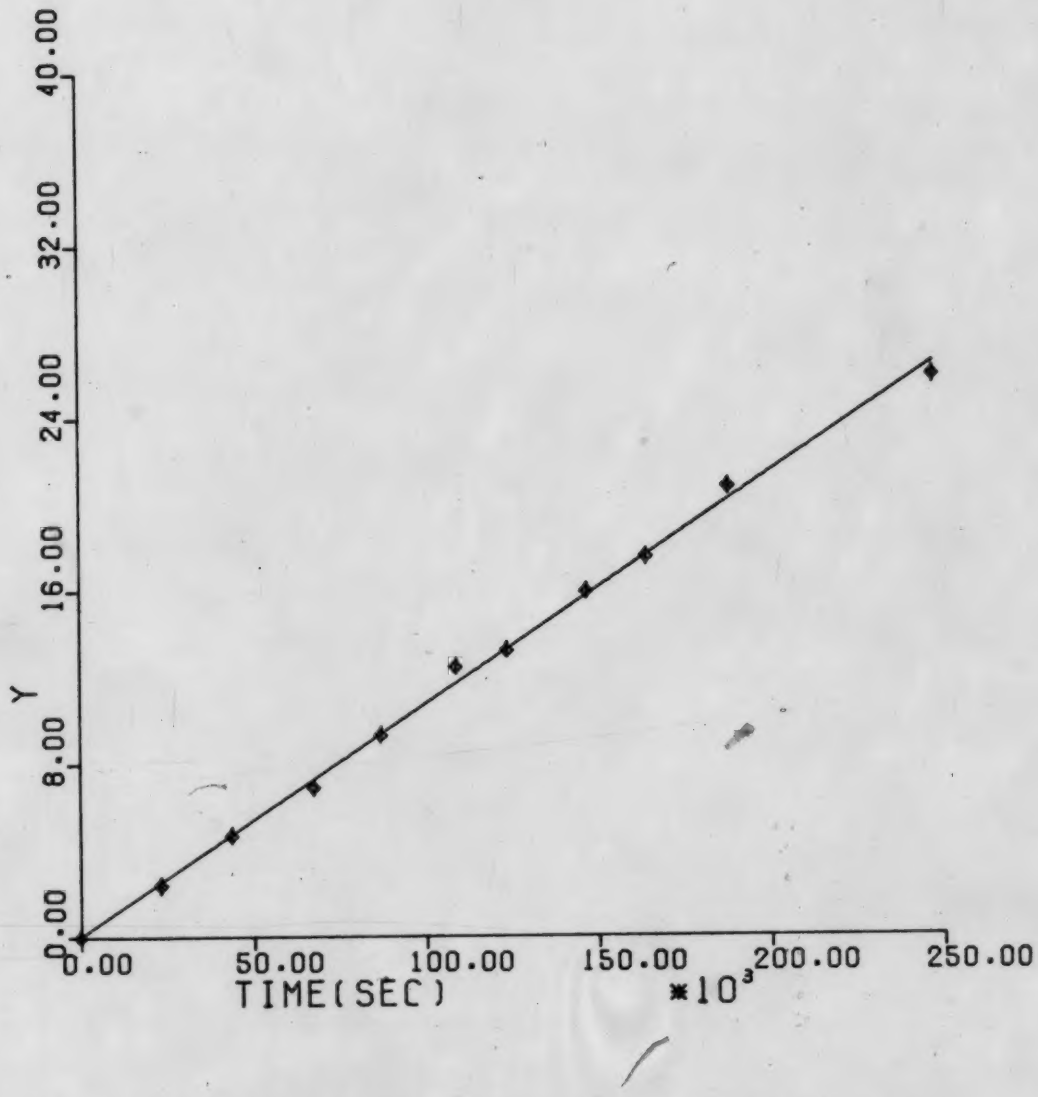


Figure 13. Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol at 90.0°. Legend as for Figure 11.

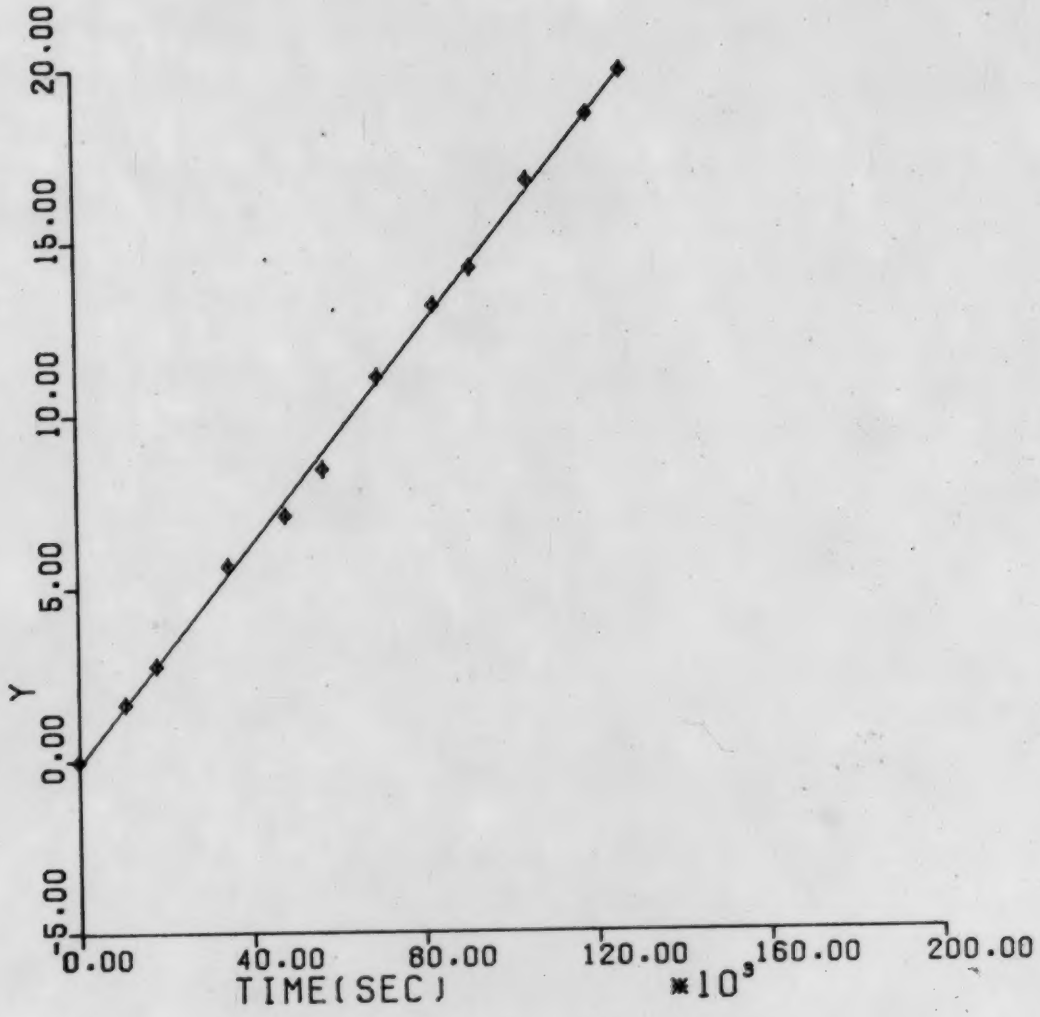


Figure 14. Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 with excess 23 in methanol at 90.0°. Legend as for Figure 11.

Table IV

Summary of Rate Constants for the Base-Catalyzed Solvolysis of 1-Methylisogramine Methiodide 23 in Methanol

Run	[KOH]	[23]	[KOH]:[23]	Temp (°C)	k_2 ($l\ mol^{-1}\ sec^{-1}$)	Figure
II-125	0.0695	0.0477	1.457	70.0	$(6.02 \pm 0.07) \times 10^{-6}$	11
II-187	0.0846	0.0474	1.785	80.0	$(2.52 \pm 0.03) \times 10^{-5}$	12
II-189	0.0877	0.0477	1.837	90.0	$(1.07 \pm 0.02) \times 10^{-4}$	13
III-103	0.0362	0.0460	0.786	90.0	$(1.58 \pm 0.02) \times 10^{-4}$	14

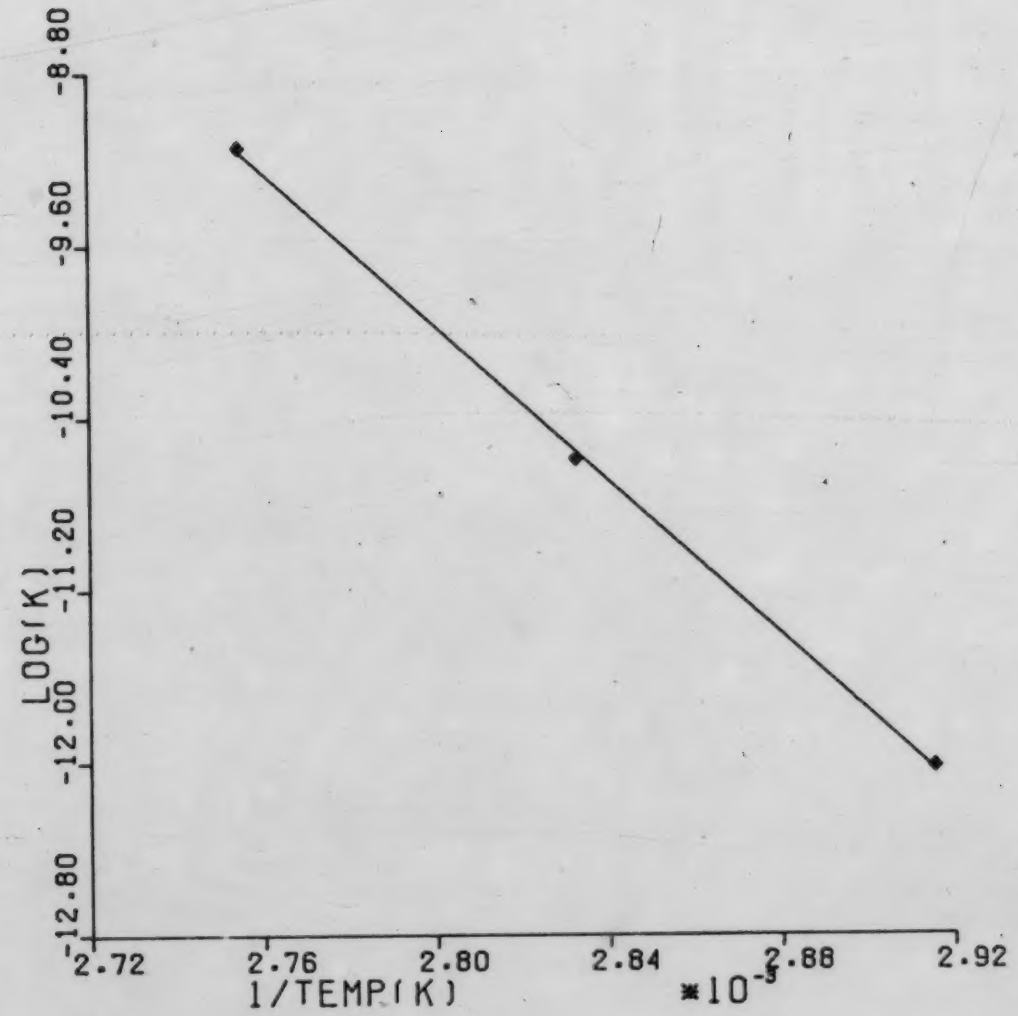


Figure 15. Plot of ln (k_2) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol. Legend as for Figure 9.

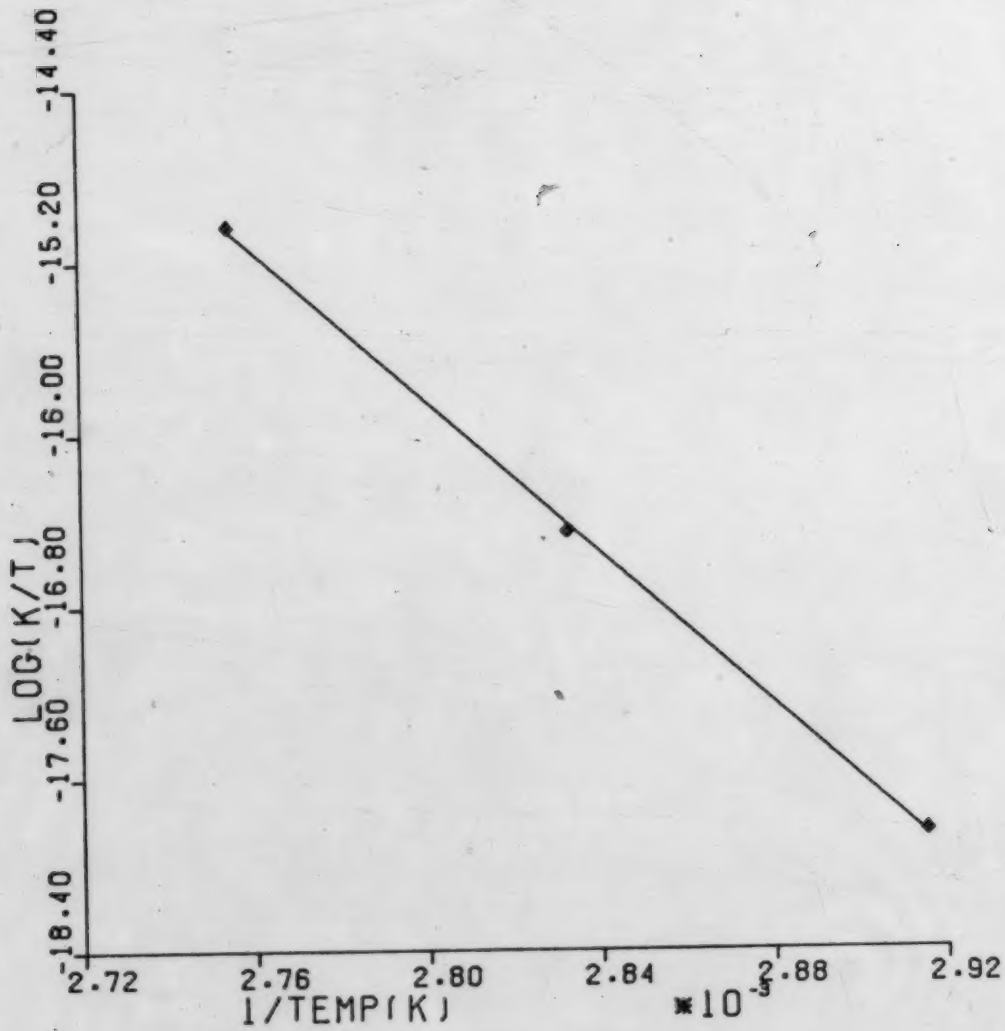


Figure 16. Plot of ln (k_2/T) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylisogramine methiodide 23 in methanol. Legend as for Figure 10.

Table V.

Table V

Activation Parameters for the Base-Catalyzed Methanolysis of 1-Methylisogramine Methiodide 23

Ea	35.6 ± 0.7 kcal/mole
$\Delta H^{\neq}$	34.9 ± 0.7 kcal/mole
$\Delta S^{\neq}$	+18.9 ± 2.0 e.u.

1-Methylindol-2-ylmethyl N-*t*-butylcarbamate 24

Synthesis

This compound was synthesized as shown in Scheme 9.

Product Run

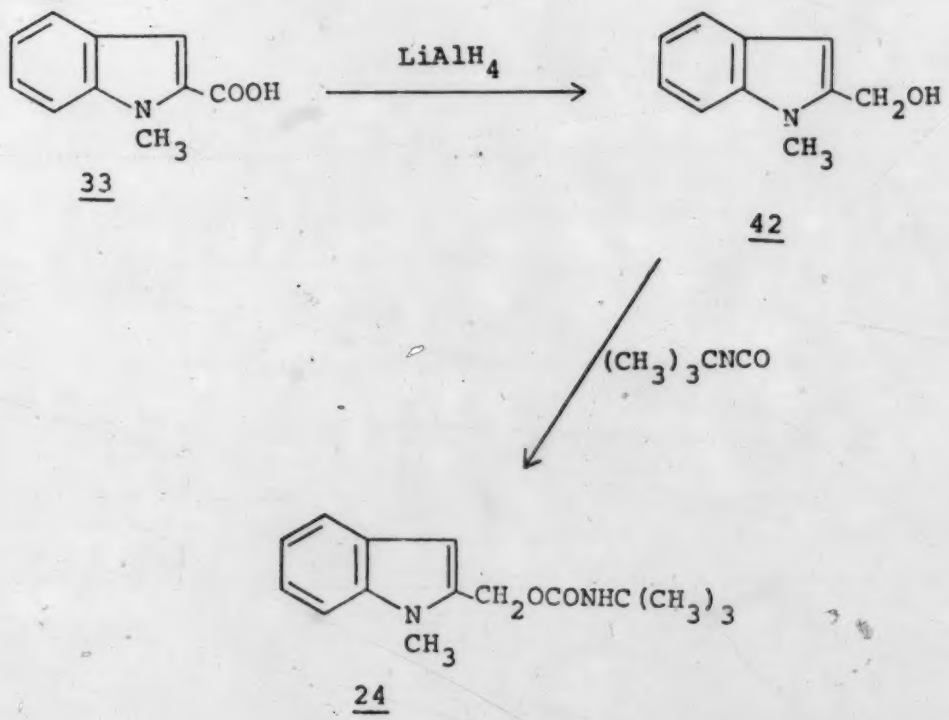
The product 41 of the solvolysis reaction was established by carrying the reaction to completion at 90.0° in an nmr tube and comparing the spectrum of the product with that of the product obtained from the solvolysis of 1-methylisogramine methiodide 23.

Kinetic Study

The reaction of Scheme 6 was studied at three temperatures: 50.0, 70.0, and 80.0°. As was the case with the corresponding gramine derivative 23, the reaction was found to follow second-order kinetics.

The results of the kinetic measurements, with rate constants being calculated from the slopes of the plots in

Scheme 9



33 42 24

Figures 17 to 19, are tabulated in Table VI.

From these observed rate constants and the plots of ln (k_2) *vs* (1/T) (Figure 20) and ln (k_2/T) *vs* (1/T) (Figure 21), the activation parameters given in Table VII were calculated.

DNA Study

By the fluorescence assay described in Chapter III there was found no indication that 24 alkylates PM2 DNA at pH 7.0 and 37°.

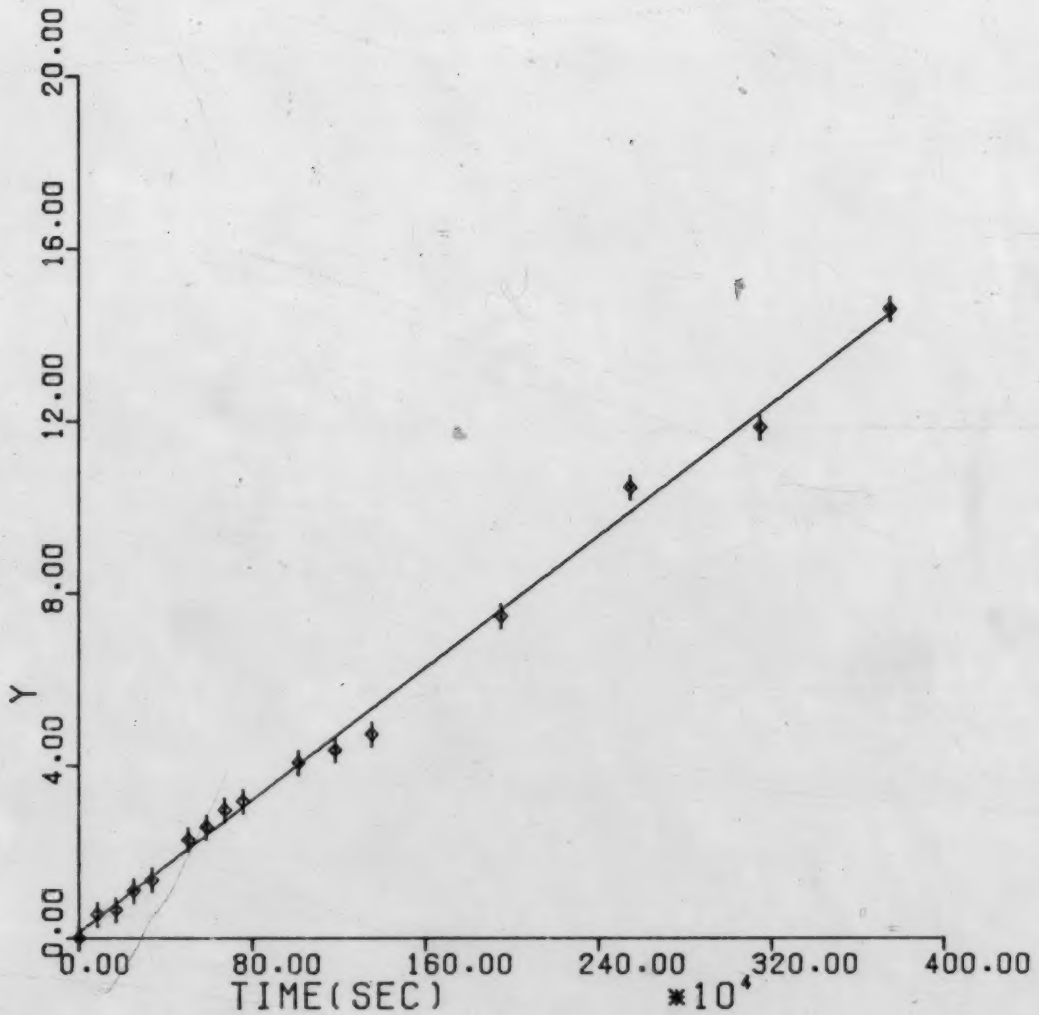


Figure 17. Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol at 50.0°. Legend as for Figure 11.

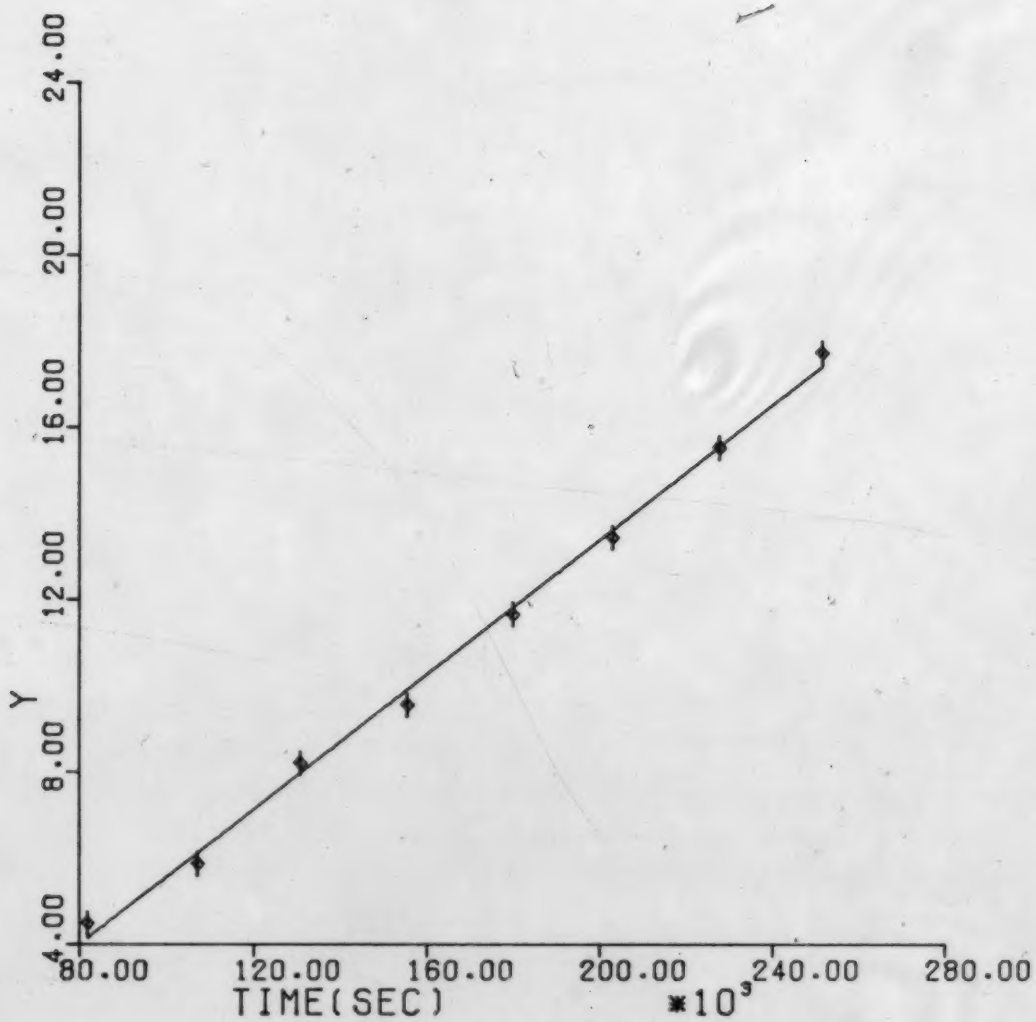


Figure 18. Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol at 70.0°. Legend as for Figure 11.

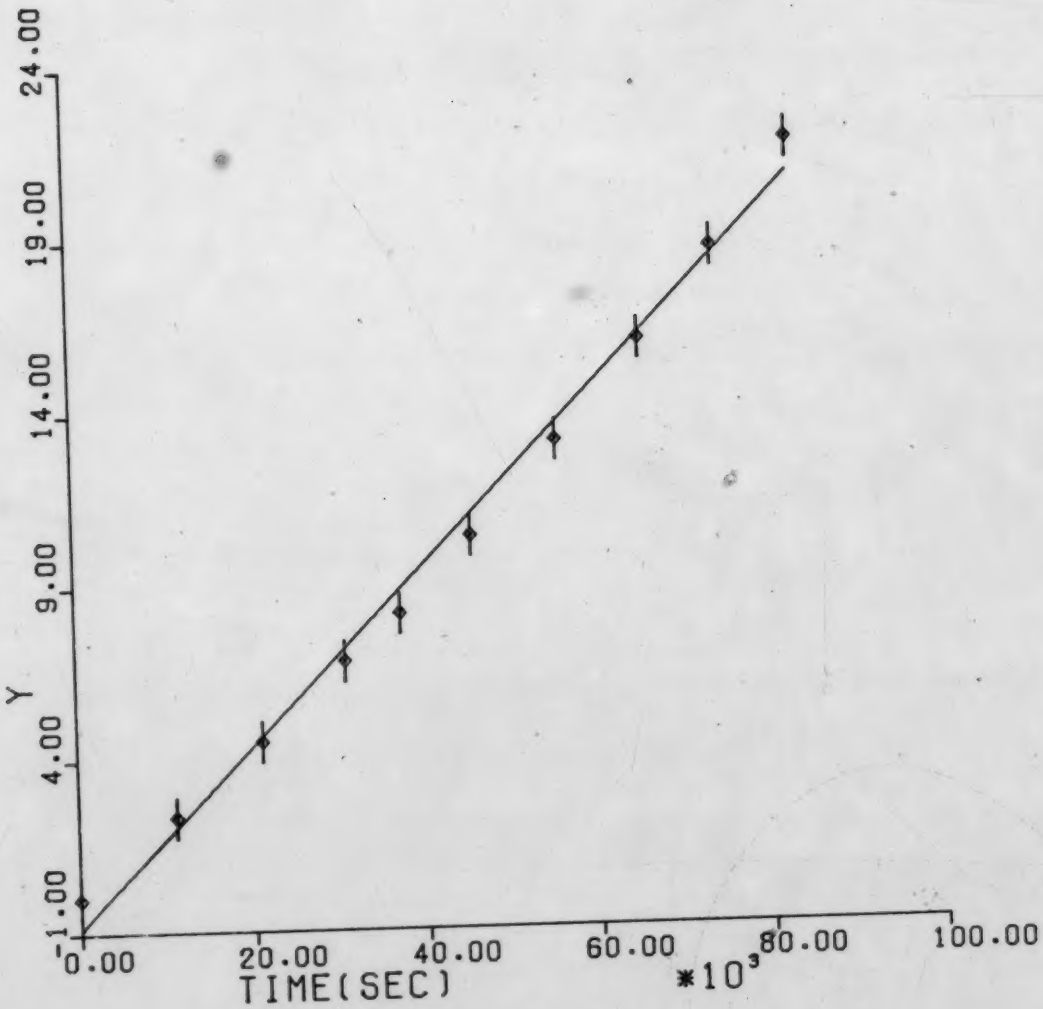


Figure 19. Plot of the integrated second-order rate expression *vs* time for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol at 80.0°. Legend as for Figure 11.

Table VI

Summary of Rate Constants for the Base-Catalyzed Solvolysis of 1-Methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in Methanol

Run	[KOH]	[24]	[KOH]:[24]	Temp (°C)	k_2(l mol^{-1} sec^{-1})	Figure
II-235	0.1341	0.0768	1.746	50.0	(3.83 ± 0.06) x 10^{-6}	17
II-239	0.1267	0.0769	1.648	70.0	(7.80 ± 0.18) x 10^{-5}	18
II-277	0.1051	0.0660	1.592	80.0	(2.59 ± 0.08) x 10^{-4}	19

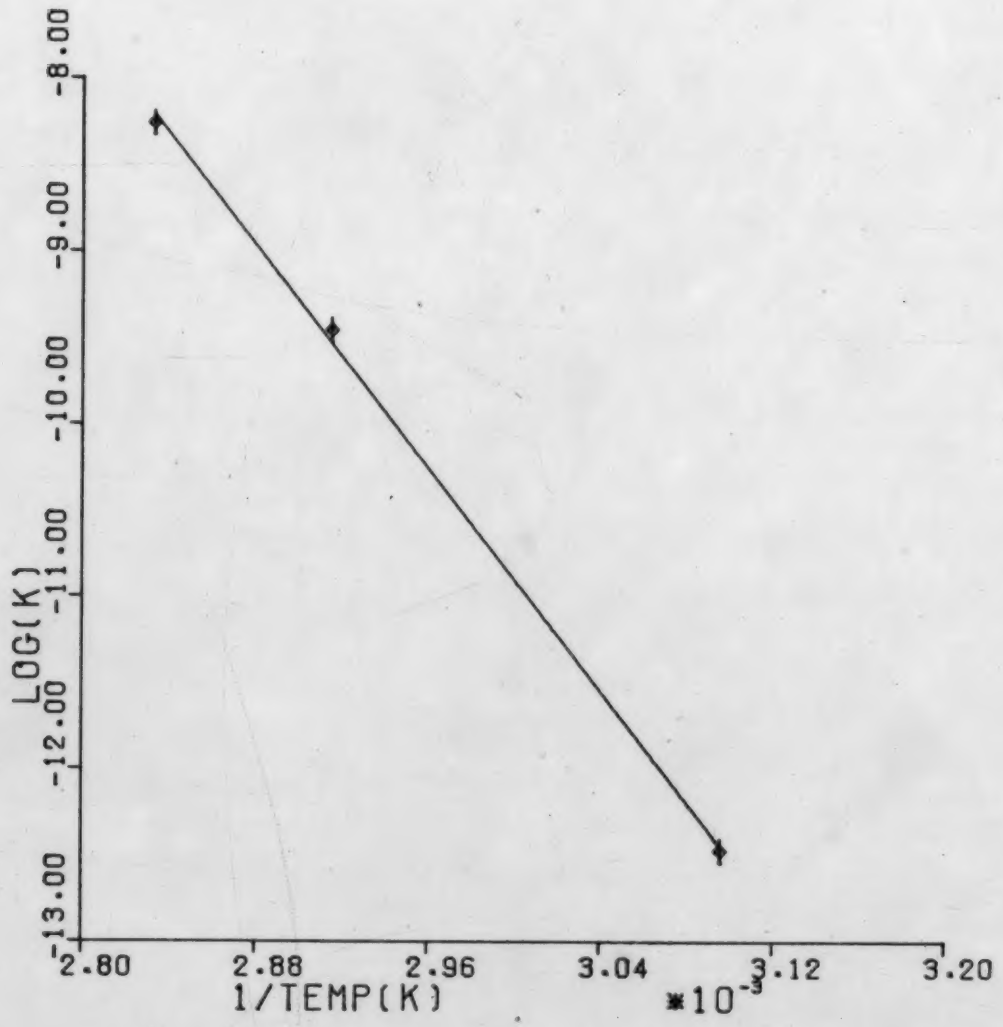


Figure 20. Plot of ln (k_2) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol. Legend as for Figure 9.

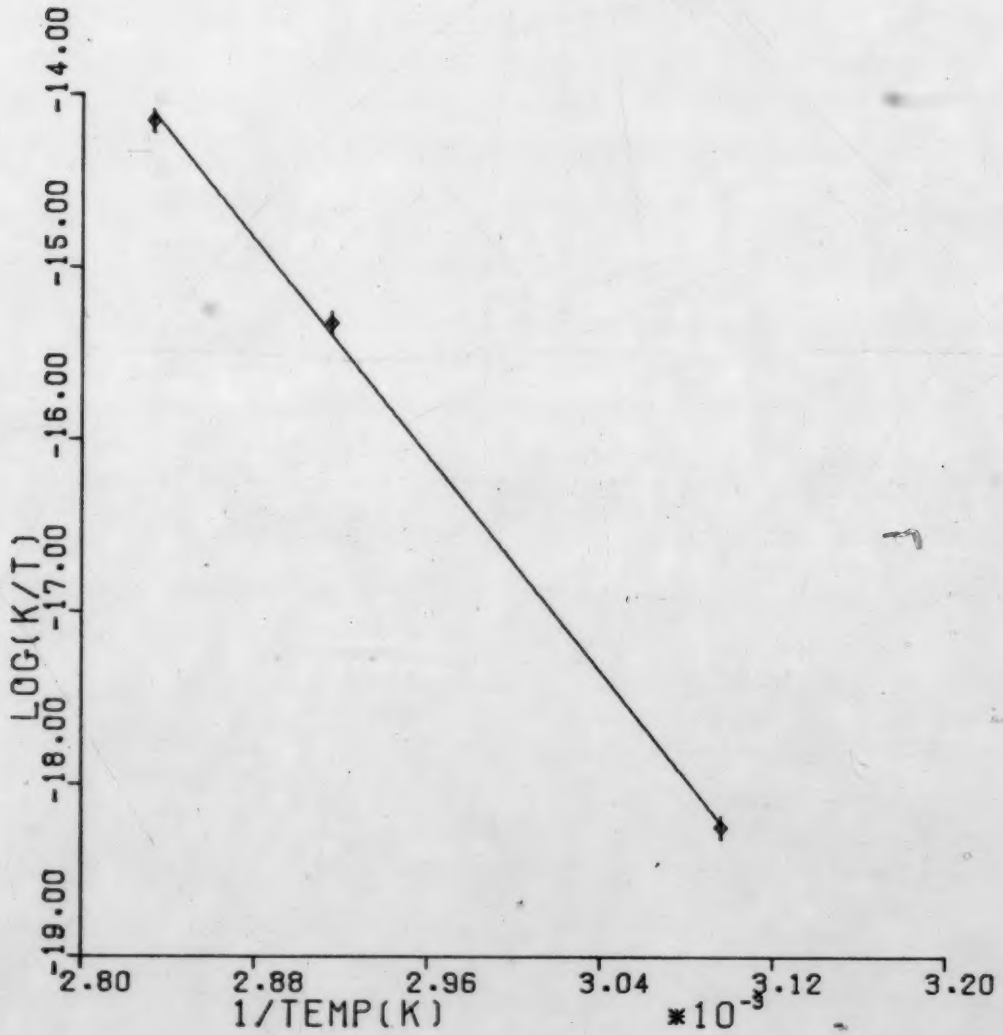


Figure 21. Plot of ln (k_2/T) *vs* (1/T) for the base-catalyzed solvolysis of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 in methanol. Legend as for Figure 10.

Table VII

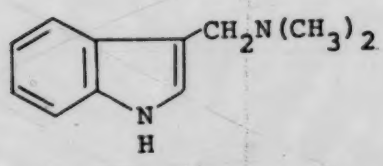
Activation Parameters for the Base-Catalyzed Methanolysis of 1-Methylindol-2-ylmethyl N-*t*-butylcarbamate 24

Ea	32.0 ± 1.1 kcal/mole
$\Delta H^{\neq}$	31.1 ± 1.0 kcal/mole
$\Delta S^{\neq}$	+13.0 ± 2.8 e.u.

Gramine Methiodide 26

Synthesis

Gramine methiodide (GMI) 26 was synthesized by the alkylation of gramine 43 with methyl iodide.

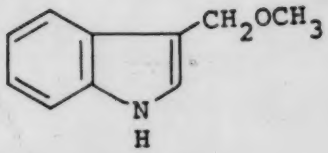


43

Product Runs

Treatment of 26 with an equivalent of sodium hydroxide in methanol gave a product of m.p. 94-95°. This value is in agreement with the value reported in the

literature[34] for the ether 44. This structure was also



44

confirmed by ir, nmr, and mass spectra.

Kinetics

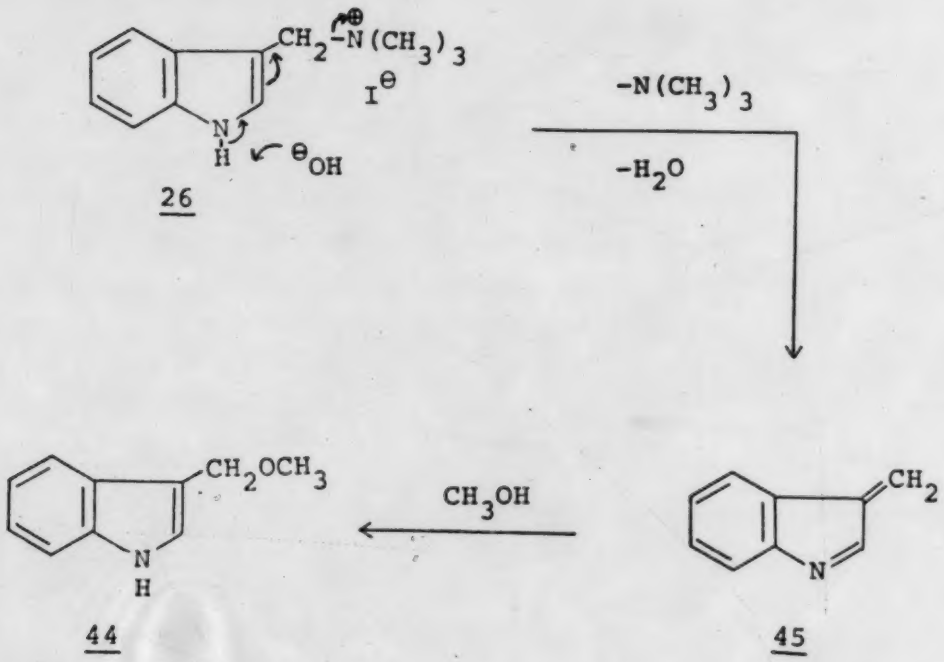
Since the reaction of 26 with base and methanol was found to be very rapid even at -78°, it was not possible to follow this reaction kinetically. Such a result is not surprising since the gramine salt possesses a very acidic NH proton which is quickly neutralized by base (Scheme 10).

DNA Studies

By the fluorescence assay techniques described in Chapter III gramine methiodide 26 was found to both alkylate and cross-link DNA. At pH 7.0 and 37°, it produced 71% cross-linking after 300 min at a concentration of 710 µg/ml; while at a concentration of 930 µg/ml at pH 8.7 and 37°, 79% cross-linking by 26 was observed after 170 min.

When the assay was performed at pH 7.0 as well as the usual pH 11.6 it was found that the before-heating

Scheme 10



26 44 45

fluorescence values were the same at equal time intervals up to 1 hr. This is similar to the behavior found for mitomycin C and is evidence against alkylation of the N-7 position of guanine.[35]

It was also found by the methods described in Chapter III that the extent of covalent cross-linking is dependent upon the guanine + cytosine (G+C) content of the DNA. These findings are tabulated in Table VIII and shown in Figure 22.

Table VIII.

Dependence of Efficiency of Covalent Cross-Linking by Gramine Methiodide 26 on the (G+C) Content of the DNA

GMI Conc. x 10^{-3} M	% (G+C)	% Cross-Linking After 230 min	Avg. Cross-Links per Molecule	Avg. Cross-Links per Nucleotide x 10^{-5}
3.3	30	49	0.673	1.77
3.3	40	65	1.050	3.46
3.3	50	41	0.528	20.6

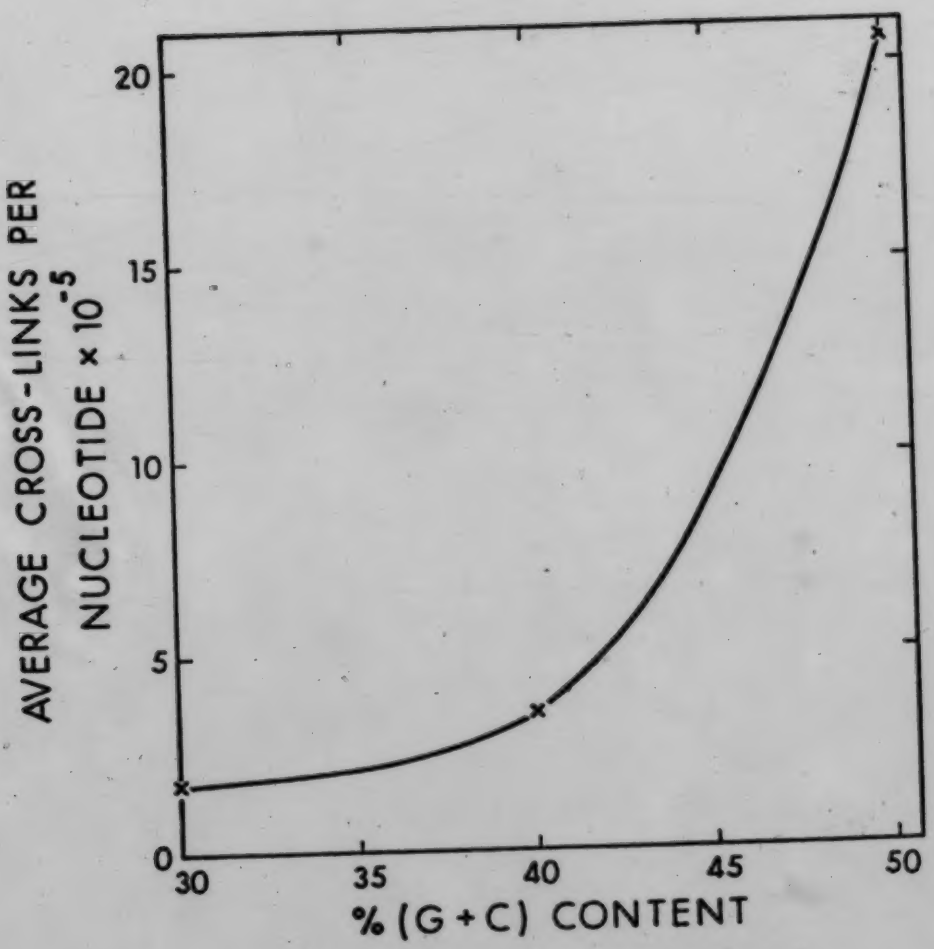


Figure 22. Dependence of efficiency of covalent cross-linking of DNA's by gramine methiodide 26 on the (G+C) content of the DNA. Reactions were performed at 37° and contained DNA at 1.25 A_{260}, 0.05 M phosphate buffer pH 8.7 and gramine methiodide 26 at 3.4×10^{-3} M.

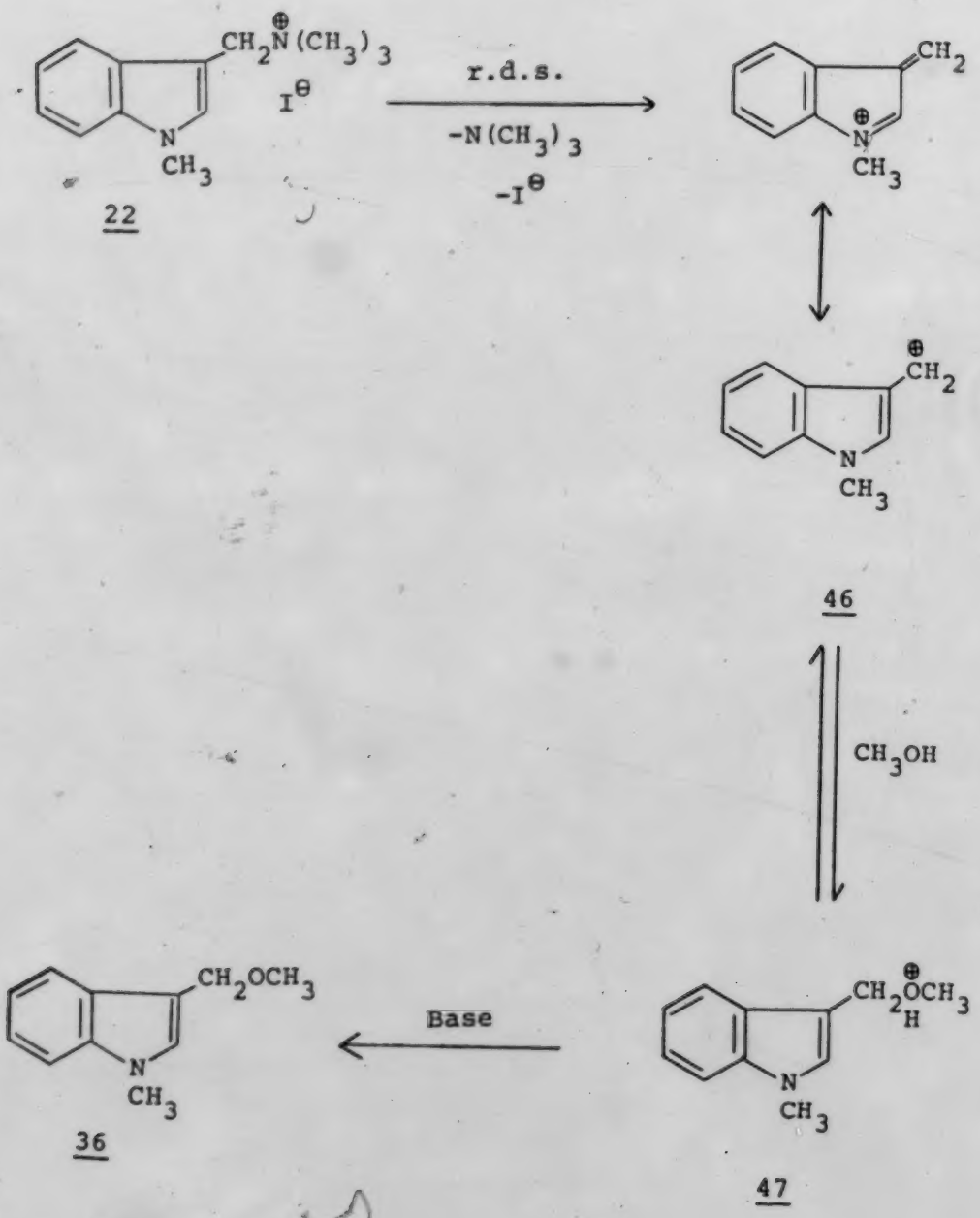
Summary and Conclusions

Gramine methiodide 26 undergoes a very rapid elimination of the elements of $(CH_3)_3\overset{\oplus}{N}H\ I^{\ominus}$ followed by an addition of a nucleophile such as solvent methanol. The process is, however, too fast to follow kinetically. Nevertheless such a mechanism could account for the method by which this compound alkylates DNA. The cross-linking process, however, requires a second alkylation site; the only other reactive locations on this molecule are the N-1 and C-2 positions. In support of the N-1 position is the lack of reactivity toward DNA of the 1-methyl derivative. Such a mechanism is also consistent with the suggestion of Murakami[36] who postulates involvement of the N-4 position of the mitomycins. The ability of gramine methiodide 26 to cross-link DNA provides evidence for the participation in the cross-linking process of the site corresponding to the methylurethane group in the mitomycins.

The solvolysis of 1-methylgramine methiodide 22 is consistent with a rate-determining unimolecular elimination of $N(CH_3)_3$ followed by a faster attack by the nucleophile. This elimination can be assisted, and the resulting intermediate stabilized, by the lone electron pair of the indole nitrogen, and would not disrupt the aromaticity of the benzene ring (Scheme 11).

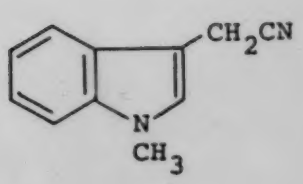
The role of the base in this reaction would seem to be more than just that of a proton sponge, since replacement of the hydroxide ion by the weaker, non-nucleophilic base,

Scheme 11

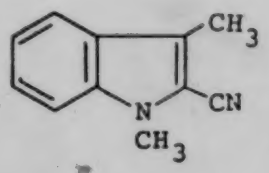

CH2N(CH3)3
I⊖
r.d.s.
-N(CH3)3
-I⊖
CH2
N
CH3
22
46
CH3OH
CH2OCH3
Base
CH2OCH3
H
47
36

2,6-lutidine, causes the reaction to proceed much less cleanly. One possible explanation might be that illustrated in Scheme 12, in which the active nucleophilic species is in fact the methoxide ion formed in a prior equilibrium with the hydroxide ion. The pKa values of water and methanol at 25° are, respectively, 15.74 and 15.5,[37] which would give a substantial proportion of available methoxide by equilibration. The situation of alkoxide being more nucleophilic than hydroxide in a mixture of both is reminiscent of the Schotten-Baumann procedure for the acylation of alcohols in alkaline media.[38]

Another possibility for the involvement of the base might be the intermediate attack by the base at the 2-position of the indole, analogous to the discovery by Snyder and Eliel[39] that treatment of 1-methylgramine methiodide 22 with aqueous sodium cyanide produced not only the expected substitution product 48, but also a small amount of the isomeric 49.

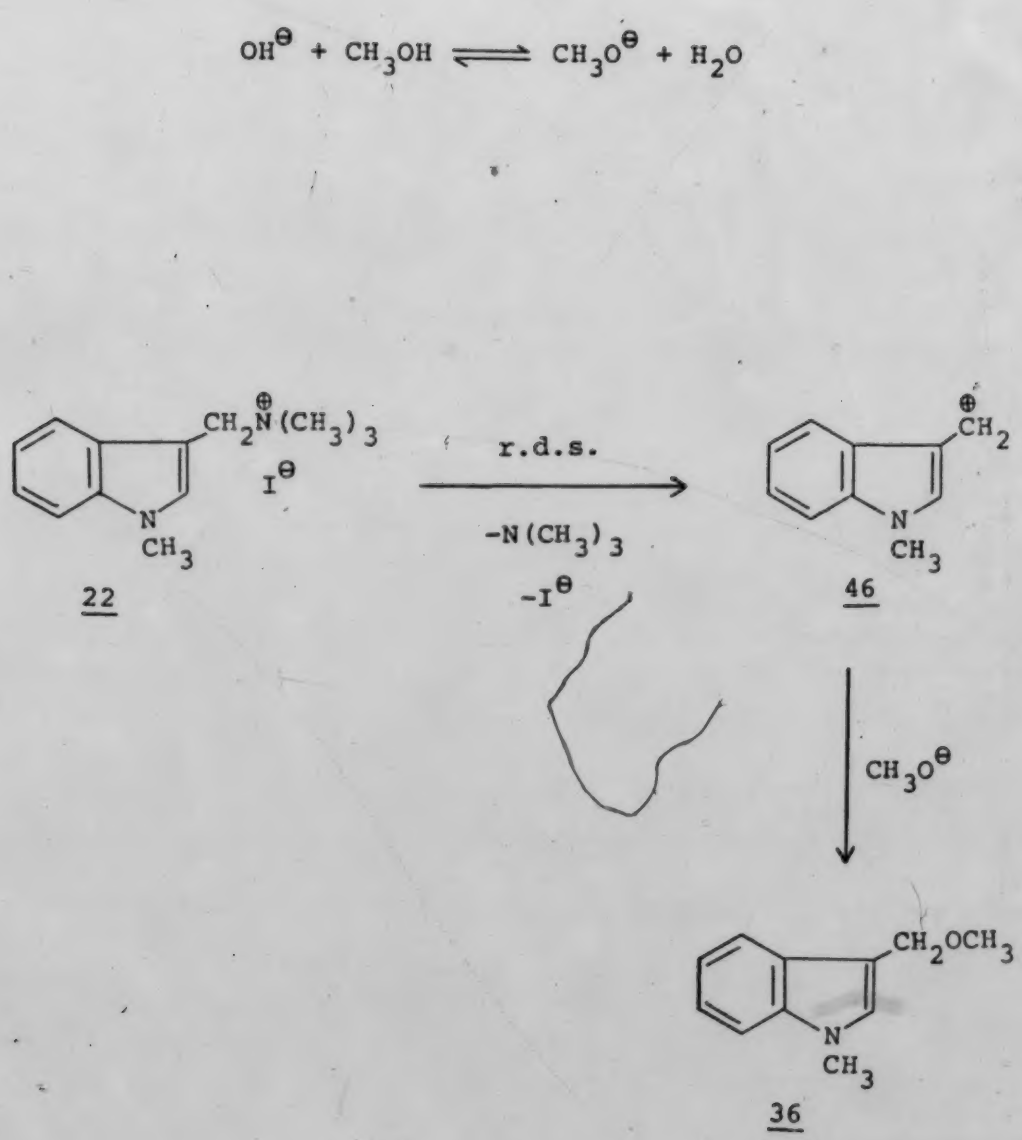


48



49

Scheme 12

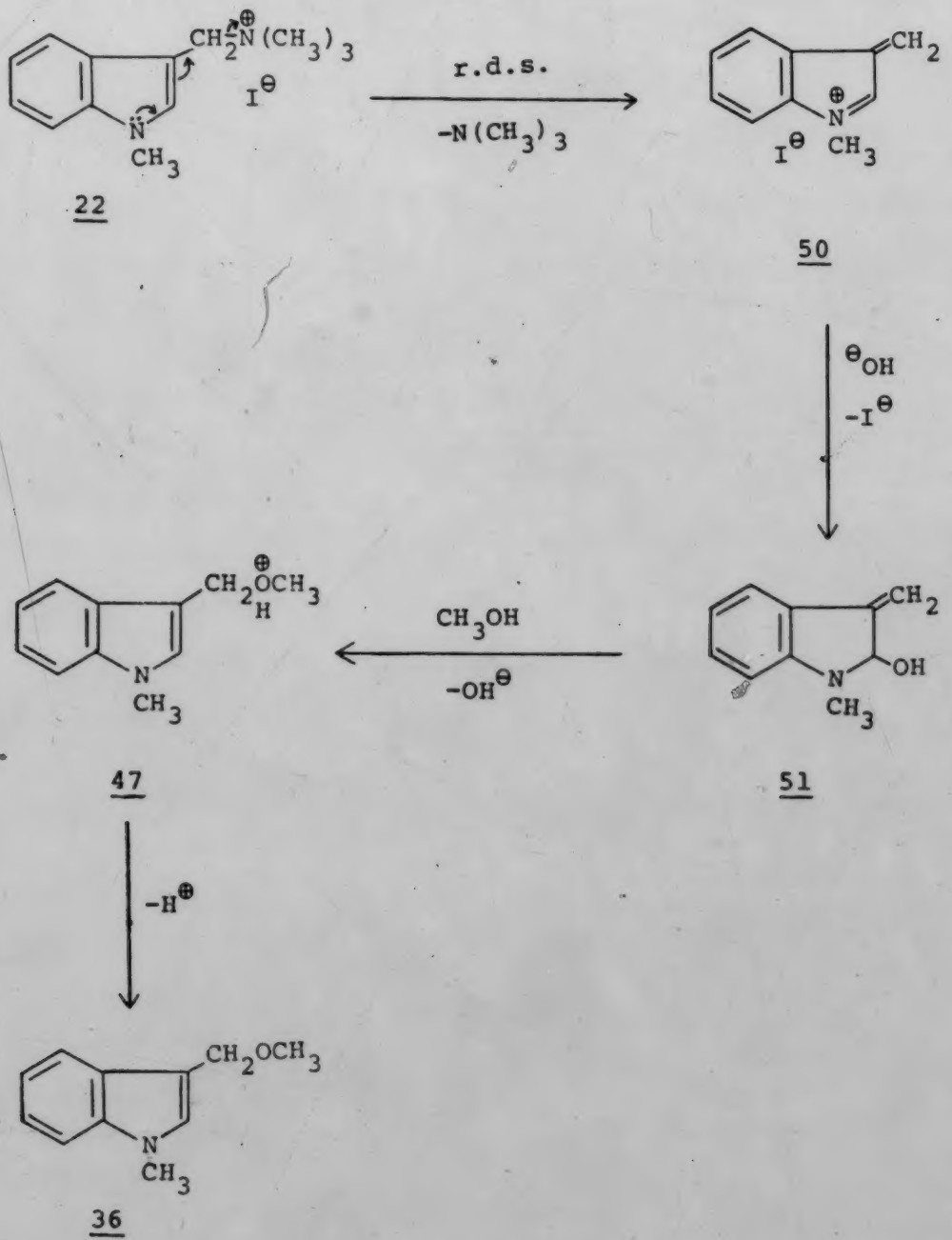

OH⊖ + CH3OH ⇌ CH3O⊖ + H2O
CH2N⊕(CH3)3
I⊖
N
CH3
22
r.d.s.
-N(CH3)3
-I⊖
CH2⊕
N
CH3
46
CH3O⊖
CH2OCH3
N
CH3
36

The analogous process, followed by an allylic displacement by the solvent methanol, is shown in Scheme 13.

The negative entropy value is unusual for unimolecular reactions which involve a molecular fragmentation with a concomitant increase in disorder. However due to the participation of the lone electron pair in assisting the fragmentation step (46), part of the delocalized 10 π electron system of the indole moiety is frozen. If the transition state leading to the formation of the intermediate 46 is structurally similar to the starting material 22, there would not be a large molecular disordering. Together with the concurrent ordering of the electrons there would be a net ordering of the system, which could well rationalize the observed value of $\Delta S^{\neq}$.

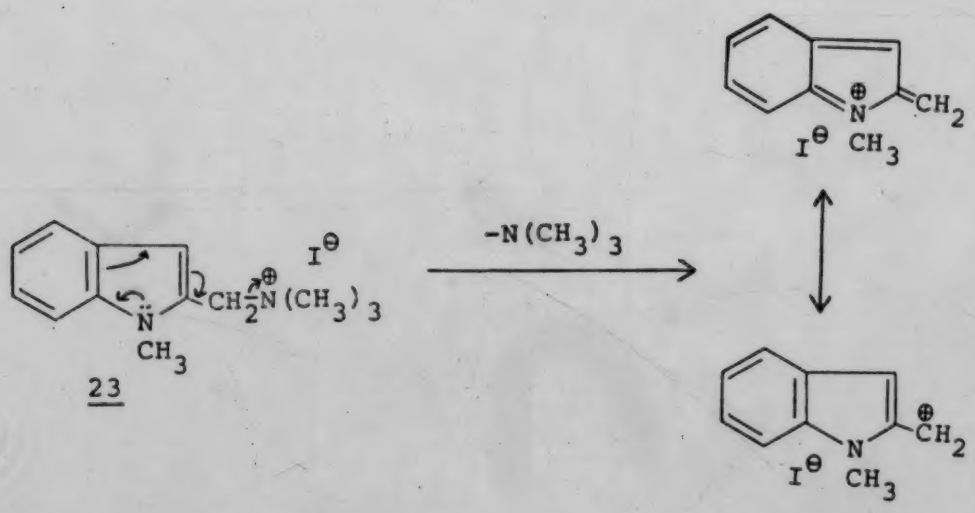
For the 1-methylisogramine system (23), the reaction is not only slower, but also exhibits second-order behavior. This would suggest that the type of electron-pair assistance analogous to that for the 1-methylgramine salt 22 and shown in Scheme 14, does not make a significant contribution to the reaction process. Since the carbenium ion process is not favored in this way, compound 23 behaves as a normal primary system and undergoes an S_N2-type of displacement. The positive value found for $\Delta S^{\neq}$ may be rationalized in terms of the partial neutralization of unlike charges taking place in the transition state. This in turn would bring about less ordering of the solvent shell surrounding the substrate ions.

Scheme 13



22 50 51 47 36

Scheme 14



23 52

The studies with the corresponding carbamate 24 show that this compound reacts more rapidly than the trimethylammonium salt and suggest that the carbamate moiety is a better leaving group for this series of reactions.

This greater reactivity of the carbamate compounds would also explain why it was not possible to synthesize the isomeric carbamate 25, in which the more reactive leaving group is located on the more reactive 3-position of the indole. All attempts to prepare such a carbamate, even under the mildest conditions, resulted in mixtures which showed extensive decomposition.

From these studies on simple model systems it would be tempting to conclude that the 10-position of the mitosanes would be more reactive than the 1-position as a

site for alkylation. Yet evidence has accumulated[13] to show that the 1-position is in fact the first to react; aziridine ring-opened derivatives will alkylate DNA, but at a much slower rate. This suggests that a rate enhancement for the 1-position due to relief of the aziridine ring strain is more than sufficient to offset the inherent comparative reactivities of these sites.

Experimental

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Grating Infrared Spectrophotometer Model 421 and generally only the principal peaks are reported. Absorption spectra were measured in either distilled water or "spectro-grade" solvents and were run on either a Beckman DB Spectrophotometer, a Gilford 250 Spectrophotometer, or a Unicam SP1700 UV Spectrophotometer. Proton magnetic resonance spectra were recorded on Varian A-60 and HA-100 analytical instruments and were generally measured on 10-15% (w/v) solutions of the compound in the appropriate deuterated solvent. The reference compound was tetramethylsilane (TMS), except for the nmr kinetic runs, where hexamethyldisilane (HMDS) was used as the reference because of its lower volatility. In either case line positions are reported in parts per million from the reference. Mass spectra were determined with Associated Electrical Industries MS2, MS9, MS12, or MS50 (DS50) mass

spectrometers. In general the ionization energy was 70 eV. Unless indicated otherwise, during work-up procedures solvents were removed under reduced pressure with a Buchi Rotavapor R rotary evaporator.

Materials

Anhydrous methanol for the product runs was prepared from commercially available methanol by treatment with magnesium methoxide and distilled as described by Fieser.[40a]

2,6-Lutidine was prepared as follows: Eastman practical grade 2,6-lutidine was dried over potassium hydroxide for 24 hr then refluxed and distilled from barium oxide as described by Fieser for the preparation of anhydrous pyridine.[40b] The center cut (138-140°) was treated with boron trifluoride etherate as described by Brown *et al.*[41]

1-Methylindole-2-carboxylic acid 33

This compound was prepared from 1-methyl-1-phenylhydrazine 30 and pyruvic acid 31 by the method of Fischer and Hess,[42] m.p. 209-210° (lit. m.p. 212°).[42]

1-Methylindole 34

Following the method of Fischer and Hess,[42] 13.9 g of 1-methylindole-2-carboxylic acid 33 was heated to 205° until the evolution of gas ceased, then cooled and steam distilled to give 3.1 g (30% yield) of the product as a brown oil. Examination of the nmr spectrum showed this to be of sufficient purity for subsequent synthetic steps.

The nmr spectrum $\delta_{TMS}(CDCl_3)$: 3.63 (s, 3H, NCH_3); 6.46 (d, J = 3 Hz, 1H, indole 3-H); 6.95 (d, J = 3 Hz, indole 2-H); 7.04-7.72 (m, 4H, aryl protons).

1-Methylgramine 35

This compound was prepared as an amber oil in 63% yield by the method of Snyder and Eliel.[43]

The nmr spectrum $\delta_{TMS}(CDCl_3)$: 2.27 (s, 6H, $N(CH_3)_2$); 3.61 (s, 2H, CH_2); 3.69 (s, 3H, indole NCH_3); 6.97 (s, 1H, indole 2-H); 7.05-7.91 (m, 4H, aryl protons).

1-Methylgramine methiodide 22

This compound was prepared according to Snyder and Eliel[43] by the alkylation of 1-methylgramine 35 with methyl iodide. The crude material was formed in 98% yield as a pink solid which was recrystallized from methanol/ether to give small white leaflets, m.p. 177-178° (dec.) (lit. inst. dec. pt. 195°).[43]

The nmr spectrum $\delta_{TMS}(DMSO\text{-}d_6)$: 3.12 (s, 9H, $^{\oplus}N(CH_3)_3$); 3.88 (s, 3H, indole NCH_3); 4.79 (s, 2H, CH_2); 7.74 (s, 1H, indole 2-H); 7.09-8.09 (m, 4H, aryl protons).

Anal. Calcd. for $C_{13}H_{19}N_2I$: C, 47.29; H, 5.80; N, 8.48; I, 38.43. Found: C, 47.31; H, 5.88; N, 8.37; I, 38.29.

Indole-1-methyl-2-(N,N-dimethyl)carboxamide 39

Patterned after the method of Schindler,[44] 9.2 g of 1-methylindole-2-carboxylic acid 33 was suspended in 135 ml of benzene (freshly distilled from sodium) under a nitrogen atmosphere. 13.5 ml of thionyl chloride distilled from

quinoline was added dropwise with magnetic stirring over a period of 45 min. The mixture was then heated to 45-50° for 1 hr, during which time the acid dissolved and the solution became dark green. The solvent and excess thionyl chloride were then removed on a high-vacuum distillation apparatus, 70 ml of dry benzene was added to the residue and this solution was added dropwise to 150 ml of benzene which had been saturated with dimethylamine gas. After the addition was completed, more dimethylamine gas was bubbled into the brown solution to ensure an excess of the amine.

After standing overnight in a refrigerator, the mixture was treated with water to dissolve the dimethylamine hydrochloride. The organic layer was removed and dried (Na_2SO_4). Removal of the solvent gave a quantitative yield of a brown oil which solidified on standing to a material with m.p. 83-84° (lit. m.p. 95°).[45]

The ir spectrum ν_{max}(film): 1620 (C=O), 1455 (C-N) cm^{-1}.

1-Methylisogramine 40

Following the general method of Schindler,[44] 6 g of lithium aluminum hydride was mixed with 110 ml of tetrahydrofuran and cooled to below 10°. Over a period of 2 hr at ambient temperature a solution of 11.0 g of indole-1-methyl-2-(N,N-dimethyl)carboxamide 39 in 90 ml of tetrahydrofuran was added dropwise with stirring. The mixture was then stirred overnight, cooled in ice water,

and carefully treated with water to decompose the unreacted lithium aluminum hydride. The mixture was filtered and the solvent removed from the filtrate to give a residue which was treated with 25 ml of 2 M sodium hydroxide solution and extracted with ether. The ether extract was dried (Na_2SO_4) and evaporated to give 9.1 g (89% yield) of product as a brown liquid.

The nmr spectrum $\delta_{TMS}(CDCl_3)$: 2.21 (s, 6H, $N(CH_3)_2$); 3.48 (s, 2H, CH_2); 3.72 (s, 3H, indole NCH_3); 6.36 (s, 1H, indole 3-H); 6.93-7.70 (m, 4H, aryl protons).

1-Methylisogramine methiodide 23

Following the procedure of Schindler,[44] 9.0 g of 1-methylisogramine 40 was alkylated with methyl iodide to give 14.0 g (89% yield) of a brownish solid. A portion of this was recrystallized from methanol/ether to give a cream-colored solid m.p. 207° (lit. m.p. 217-219°).[44]

The nmr spectrum $\delta_{TMS}(DMSO\text{-}d_6)$: 3.16 (s, 9H, $^{\oplus}N(CH_3)_3$); 3.88 (s, 3H, indole NCH_3); 4.92 (s, 2H, CH_2); 6.90 (s, 1H, indole 3-H); 7.00-7.87 (m, 4H, aryl protons).

Anal. Calcd. for $C_{13}H_{19}N_2I$: C, 47.29; H, 5.80; N, 8.48; I, 38.43. Found: C, 47.04; H, 5.82; N, 8.51; I, 38.80.

2-Hydroxymethyl-1-methylindole 42

A solution of 5 g of 1-methylindole-2-carboxylic acid 33 in 100 ml of tetrahydrofuran was treated cautiously with 3.5 g of lithium aluminum hydride, then refluxed for 1 hr. The mixture was then cooled and treated with

ethyl acetate and then water to decompose the excess of lithium aluminum hydride. The resulting mixture was filtered and the filtrate dried (Na_2SO_4). Upon evaporation of the solvent this solution yielded 4.0 g (87% yield) of a light yellow solid m.p. 92-93° (lit. m.p. 108-110°).[46] The nmr spectrum indicated a purity sufficient for subsequent steps.

The nmr spectrum $\delta_{TMS}(CDCl_3)$: 1.95 (br, 1H, OH); 3.67 (s, 3H, CH_3); 4.67 (s, 2H, CH_2); 6.37 (s, 1H, indole 3-H); 6.96-7.68 (m, 4H, aryl protons).

1-Methylindol-2-ylmethyl N-*t*-butylcarbamate 24

A mixture of 1.50 g of 2-hydroxymethyl-1-methylindole 22 and 2.3 ml of *t*-butyl isocyanate was warmed on a steam bath until all the solid material had dissolved. The solution was then cooled overnight in a refrigerator, the resulting precipitate collected, washed with petroleum ether (b.p. 60-110°), and evaporated to give 1.51 g of a dark brown material. Extraction of this substance with ether left a brown earthy residue and gave a solution from which 0.78 g (32% yield) of a cream-colored material precipitated upon concentration. This substance was recrystallized from either acetone/water or ether/*n*-pentane to give crystals, m.p. 103°.

The ir spectrum $\nu_{max}(CHCl_3)$: 3440, 2970, 1720 (carbamate C=O), 1495, 1460, 1390, 1360, 1335, 1315, 1255, 1135, 1065 cm^{-1}.

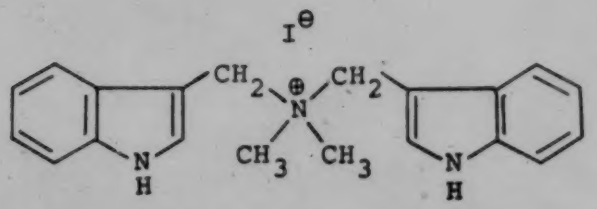
The nmr spectrum $\delta_{TMS}(CDCl_3)$: 1.33 (s, 9H,

$C(CH_3)_3$); 3.74 (s, 3H, NCH_3); 4.47-4.95 (br, 1H, NH); 5.23 (s, 2H, CH_2); 6.57 (s, 1H, indole 3-H); 6.93-7.70 (m, 4H, aryl protons).

Anal. Calcd. for $C_{15}H_{20}N_2O_2$ [mol. wt. 260.1524]: C, 69.20; H, 7.74; N, 10.76; O, 12.29. Found [(mass spectrum) 260.1519]: C, 69.32; H, 7.89; N, 10.88; O, 12.85.

Gramine methiodide 26

The method of Geissman and Armen[34] was found to give a sticky material containing impurities of dimethyldi-(3-indolylmethyl)ammonium iodide 53, as evidenced by the nmr spectrum (singlets at 2.85 and 7.30 δ_{TMS} in $CDCl_3$).



53

An improved method involved first dissolving 2.75 g of gramine 43 in 300 ml of ether and adding this nearly saturated solution dropwise with stirring to 100 g of methyl iodide. As the addition proceeded a white-to-pale-yellow solid precipitated from the reaction mixture in quantitative yield. The product was recrystallized from

methanol/ether as white crystals, m.p. 168-170° (lit. m.p. 168-169°).[34]

The nmr spectrum δ_{TMS}(DMSO-d_6): 3.12 (s, 9H, $^{\oplus}N(CH_3)_3$); 4.80 (s, 2H, CH_2); 7.76 (d, J = 2.5 Hz, 1H, indole 2-H); 7.05-8.03 (m, 4H, aryl protons); 11.57 (br, 1H, NH).

Anal. Calcd. for $C_{12}H_{17}N_2I$: C, 45.58; H, 5.42; N, 8.86; I, 40.14. Found: C, 45.63; H, 5.67; N, 8.65; I, 40.14.

Treatment of 1-methylgramine methiodide 22 with sodium hydroxide in methanol

A solution of 1.495 g (4.53 mmole) of 1-methylgramine methiodide 22 and 0.181 g (4.53 mmole) of sodium hydroxide in 100 ml of anhydrous methanol was placed in a stoppered volumetric flask and suspended for 209 hr in an oil bath thermostated at 70.0°. The solvent was then removed *in vacuo* and the organic portion of the residue extracted with ether. Recovery was 0.790 g (4.51 mmole, 99.6%) of 3-methoxymethyl-1-methylindole 36 as a brown oil which became hard and vitreous on prolonged standing.

The ir spectrum ν_{max}($CHCl_3$): 2900, 1635, 1590, 1450, 1350, 1305, 1130, 990 cm^{-1}.

The nmr spectrum δ_{TMS}($CDCl_3$): 3.36 (s, 3H, OCH_3); 3.67 (s, 3H, NCH_3); 4.63 (s, 2H, CH_2); 6.99 (s, 1H, indole 2-H); 7.07-7.82 (m, 4H, aryl protons).

Mol. wt. Calcd. for $C_{11}H_{13}NO$: 175.0997. Found

(mass spectrum): 175.0993.

Treatment of 1-methylgramine methiodide 22 in methanol in the absence of sodium hydroxide

A solution of 0.147 g (0.445 mmole) of 1-methylgramine methiodide 22 in 10 ml of anhydrous methanol was stoppered in a 25 ml pear-shaped flask and suspended for 311 hr in an oil bath at 70.0°. The ether extract of the evaporation residue gave a dark material which appeared (nmr spectrum) to be extensively decomposed. There was no indication of the presence of the ether 36.

Treatment of 1-methylgramine methiodide 22 with sodium hydroxide in water

A solution of 0.159 g (0.483 mmole) of 1-methylgramine methiodide 22 and 0.021 g (0.531 mmole) of sodium hydroxide in 10 ml of distilled water was stoppered in a 25 ml pear-shaped flask and suspended for 48 hr in an oil bath at 70.0°. The ether extract of the residue after evaporation gave 60 mg of an orange oil. The mass spectrum (m = 274) and the aryl:CH_2 and aryl:CH_3 ratios of the nmr integrations indicated that this product was di-(1-methylindolyl)-methane 37.

Treatment of 1-methylgramine methiodide 22 with 2,6-lutidine in methanol

A solution of 0.115 g (0.347 mmole) of 1-methylgramine methiodide 22 and 0.044 g (0.409 mmole) of 2,6-lutidine in 5 ml of anhydrous methanol was sealed in a test tube and placed into a 70.0° oil bath for 259 hr. The

workup as described above gave only 1 mg of the ether 36 (mass spectrum: m = 175) plus 50 mg of solid materials which could not be identified.

Treatment of 1-methylgramine methiodide 22 with sodium methoxide in *t*-butyl alcohol

0.136 g (0.412 mmole) of 1-methylgramine methiodide 22 and 0.024 g (0.437 mmole) of sodium methoxide were suspended in 10 ml of *t*-butyl alcohol and placed in a 70.0° oil bath for 187 hr. From the workup was isolated 20 mg of a yellowish oil which from the nmr spectrum and mass spectrum (m = 217) was identified as 3-*t*-butoxymethyl-1-methylindole 38.

The nmr spectrum $\delta_{TMS}(CDCl_3)$: 1.35 (s, 9H, $C(CH_3)_3$); 3.72 (s, 3H, NCH_3); 4.65 (s, 2H, CH_2); 6.97-7.36 (br m, 5H, aryl plus indole 2-H protons).

Treatment of 1-methylisogramine methiodide 23 with sodium hydroxide in methanol

A solution of 1.257 g (3.81 mmole) of 1-methylisogramine methiodide 23 and 0.152 g (3.81 mmole) of sodium hydroxide in 100 ml of anhydrous methanol was placed in a 70.0° oil bath for 425 hr. The solution was then evaporated to dryness and extracted with ether to give 0.19 g (31% yield) of 2-methoxymethyl-1-methylindole 41 as a yellow solid, m.p. 44-47°. 69% of unreacted 23 was also obtained from the ether-insoluble residue.

The ir spectrum $\nu_{max}(CHCl_3)$: 2930, 1660, 1605, 1460, 1395, 1335, 1310, 1135, 1075, 905 cm^{-1}.

The nmr spectrum $\delta_{TMS}(CDCl_3)$: 3.31 (s, 3H, OCH_3); 3.73 (s, 3H, NCH_3); 4.57 (s, 2H, CH_2); 6.46 (s, 1H, indole 3-H); 6.87-7.73 (m, 4H, aryl protons).

Mol. wt. Calcd. for $C_{11}H_{13}NO$: 175.0997. Found (mass spectrum): 175.0994.

Treatment of 1-methylisogramine methiodide 23 with sodium hydroxide in water

A solution of 0.162 g (0.490 mmole) of 1-methylisogramine methiodide 23 and 0.030 g (0.739 mmole) of sodium hydroxide in 10 ml of distilled water was stoppered in a 25 ml pear-shaped flask and suspended in an oil bath thermostated at 90.0° for 160 hr. After the workup procedure described above 0.132 g (0.400 mmole, 82% yield) of the starting material was recovered. Only a trace of ether extract was obtained; this material could not be characterized.

Treatment of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 with potassium hydroxide in methanol-d_4

20.00 mg (0.0768 mmole) of 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 and 8.18 mg of 86.5% potassium hydroxide (0.126 mmole) were sealed in an nmr tube with 1.0 ml of methanol-d_4 containing one drop of HMDS. The nmr spectrum was recorded and the tube was then placed in a 90.0° oil bath for 164 hr. After this the nmr spectrum was again recorded, and found to be that of *t*-butylamine plus 2-(trideuteromethoxy)methyl-1-methylindole 28.

The nmr spectrum before heating $\delta_{HMDS}(CD_3OD)$: 1.25 (s, 9H, $C(CH_3)_3$); 3.66 (s, 3H, NCH_3); 4.90 (s, exchangeable protons); 5.12 (s, 2H, CH_2); 6.41 (s, 1H, indole 3-H); 6.82-7.52 (m, 4H, aryl protons).

The nmr spectrum after heating $\delta_{HMDS}(CD_3OD)$: 1.24 (s, 9H, $C(CH_3)_3$); 3.72 (s, 3H, NCH_3); 4.90 (s, exchangeable protons); 4.68 (s, 2H, CH_2); 6.32 (s, 1H, indole 3-H); 6.82-7.50 (m, 4H, aryl protons).

Treatment of gramine methiodide 26 with sodium hydroxide in methanol

A solution of 1.405 g (4.44 mmole) of gramine methiodide 26 and 0.178 g (4.45 mmole) of sodium hydroxide in 100 ml of anhydrous methanol was placed in a pressure bottle in an oil bath at 70.0° for 99 hr. The solvent was then removed and the residue extracted with ether to give 0.700 g (4.34 mmole, 98% yield) of 3-methoxymethylindole 44 as a solid, m.p. 94-95° after recrystallization from benzene/*n*-pentane (lit. m.p. 99-100°).[47] Thin-layer chromatography on silica gel with either benzene or benzene:*n*-pentane 10:90 as the developing solvent showed a single component. The mass spectrum showed a parent ion at m/e = 161.

The ir spectrum $\nu_{max}(CHCl_3)$: 3475, 3000, 2930, 1455, 1415, 1335, 1085 cm^{-1}.

The nmr spectrum $\delta_{TMS}(CDCl_3)$: 3.40 (s, 3H, OCH_3); 4.67 (s, 2H, CH_2); 6.99 (d, J = 2.5 Hz, 1H, indole 2-H); 7.08-7.89 (m, 4H, aryl protons); 8.21 (br, 1H, NH).

Methanolysis of gramine methiodide 26 in the absence of base

A solution of 0.646 g (2.04 mmole) of gramine methiodide 26 in 100 ml of anhydrous methanol was sealed in a pressure bottle and placed in a 70.0° oil bath for 121 hr. The product was a brown intractable material.

Kinetic Studies

For each kinetic run, the salt or carbamate ester was weighed into a clean nmr tube. The calculated amount of 86.5% potassium hydroxide was dissolved in about 0.5 ml of CD_3OD in a 1 ml volumetric flask. The flask was then filled to the mark with CD_3OD and two drops of HMDS as an internal standard. The contents of the volumetric flask were transferred to the nmr tube which was then sealed.

The slower reactions were conducted in a constant temperature bath with the tube being quenched in ice water prior to the spectrum recordings; for the faster reactions, the reaction was conducted directly in the heated probe of the nmr instrument. The technique consisted of monitoring the integrals of the N-trimethyl signals for the salts, and the methylene signal for the carbamate ester. The per cent of reaction at timed intervals was established by calculating the ratio of the N-trimethyl (or methylene) integral due to the reaction product, to the total N-trimethyl (or methylene) integral.

Except for the faster runs, integrations of the spectra were usually run in duplicate or triplicate, and the results averaged to reduce random fluctuations.

DNA Studies

The fluorescence assay techniques used for the investigation of the interaction of these indole derivatives are described in detail in Chapter III.

The experiment with 1-methylgramine methiodide 22 was carried out on a 150 µl scale at 37°. The reaction mixtures contained PM2 CCC-DNA at 0.67 A_{260}, 0.07 M phosphate buffer pH 10.3, and 1-methylgramine methiodide 22 at concentrations of 6.7×10^{-4} M, 6.7×10^{-3}M, or 1.3×10^{-2} M. 20 µl aliquots were withdrawn at timed intervals, up to a total of 1.5 hr for the two solutions with the lower concentrations of 22, up to a total of 20 hr for the solution most concentrated in 22. A control reaction containing no 1-methylgramine methiodide 22 was also run. Over the time periods studied there was no drop in the after-heating fluorescence of the samples. (Alkylation of the DNA would have produced a drop in this value).

The experiment with 1-methylindol-2-ylmethyl N-*t*-butylcarbamate 24 was carried out on a 200 µl scale at 37°. The reaction mixtures contained PM2 CCC-DNA at 0.50 A_{260}, 0.05 M phosphate buffer pH 7.0, and 24 at concentrations of 5.0×10^{-3} M or 1.0×10^{-2} M (made from a stock solution of 24 at a concentration of 2.0×10^{-2} M in acetonitrile). A control reaction containing 50% acetonitrile by volume, but no 24, was also run. 20 µl aliquots were withdrawn at timed intervals, these showed no drop in the after-heating fluorescence after 16 hr.

The alkylation experiments with gramine methiodide 26 were conducted on a 200 µl scale at 37°. The first mixtures contained PM2 CCC-DNA at 0.5 A_{260}, 0.05 M phosphate buffer pH 7.0, and gramine methiodide 26 at concentrations of 5.5×10^{-3} M or 1.1×10^{-2} M. The second mixtures contained PM2 CCC-DNA at 1.0 A_{260}, 0.05 M phosphate buffer pH 7.0, and gramine methiodide 26 at concentrations of 7.0×10^{-3} M or 1.4×10^{-2} M. In both cases a control experiment containing no 26 was run. 20 µl samples were withdrawn at timed intervals over a period of 130 min. In all experiments the before-heating fluorescence showed an initial drop for about the first 30 min, followed by a rise, suggestive of alkylation followed by slower strand scission. The after-heating fluorescence values showed a gradual rise, suggestive of cross-linking.

The cross-linking of DNA by 26 was confirmed by experiments with λ DNA run on a 200 µl scale at 37°. Reaction mixtures contained λ DNA at 1.2 A_{260}, 0.05 M phosphate buffer pH 7.0 or 8.7, and 26 at concentrations of 2.2×10^{-4} M or 2.2×10^{-3} M for pH 7.0 experiments, and 2.9×10^{-4} M or 2.9×10^{-3} M for pH 8.7 experiments. For both pH values a control experiment was run with no 26. At pH 7.0 the ratio of after-heating fluorescence to before-heating fluorescence (which is proportional to the per cent of cross-linking) reached a maximum after 300 min, indicating 71% cross-linking for the higher concentration of the gramine salt, and 13% cross-linking for the lower con-

centration. At pH 8.7 the maximum ratio was reached after only 170 min, giving 79% and 18% cross-linking for the more and less concentrated solutions, respectively. In both cases the control experiments showed no cross-linking.

A 200 µl solution containing λ DNA at 1.2 A_{260}, 0.05 M phosphate buffer pH 8.7 and gramine methiodide 26 at a concentration of 3.4×10^{-3} M was incubated at 37° and analyzed by the ethidium bromide assay at pH values of 11.6 and 7.0; 15 µl aliquots were withdrawn at timed intervals up to a total of 2 hr. The before-heating fluorescence of the samples dropped with time, indicative of alkylation of the DNA. Moreover, these fluorescence readings were the same at the two pH values of the assay, thus providing evidence against the N-7 alkylation of guanine.[35]

For the base-dependence studies, 200 µl solutions were prepared containing the DNA at 1.25 A_{260}, 0.05 M phosphate buffer pH 8.7, and gramine methiodide 26 at 3.4×10^{-3} M. The reactions were incubated at 37°, with aliquots withdrawn at timed intervals to a total of 230 min. The extent of cross-linking was determined by the ethidium bromide assay.

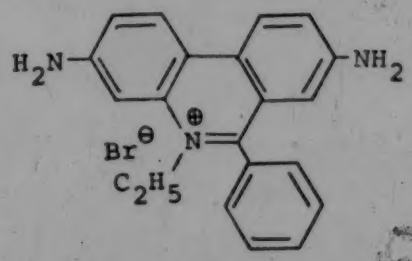
CHAPTER III

STUDIES RELATED TO THE MECHANISM OF ACTION OF MITOMYCIN B

Although mitomycin B is potent as both an antibiotic and an antitumor agent, little is known about its mode of action, except by analogy with other members of the mitosane family. For example, mitomycin C exhibits two-fold behavior toward the genetic material of the cell: it alkylates and cross-links DNA, and it causes degradation of the DNA.

We have undertaken an investigation of this behavior of mitomycin B analogous to the studies which have been reported for mitomycin C.[12,13] A correlation between the behavior of these two mitosanes and their structural differences will help to clarify many of the aspects of their mode of action which are not completely understood.

The technique employed to study the behavior of mitomycin B is an assay based on the enhancement of fluorescence of the trypanocidal dye, ethidium bromide 54 when it has interacted specifically with double-stranded DNA.



54

Using variations of such an assay it has been possible to estimate:

1. direct cross-linking of DNA,
2. sequential cross-linking of DNA,
3. alkylation of DNA without necessarily cross-linking,
4. induction of single strand scission in covalently closed circular DNA (CCC-DNA) and its inhibition by radical scavengers and certain enzymes,
5. simultaneous cross-linking and strand scission.

In addition to the fluorescence assays, electrochemical studies were also carried out in order to investigate the mechanism of the reduction of the mitosanes which is required for the biological activation of these compounds.

The Ethidium Bromide Fluorescence Assay

Le Pecq and Paoletti[48] observed that ethidium bromide 54 shows a very pronounced increase in fluorescence when bound to bihelical nucleic acids; however studies with homopolymer rA_n showed that there was no enhancement of fluorescence with this polymer, indicating that only bihelical polynucleotides produce the enhancement of fluorescence. Morgan and Paetkau[49] have also confirmed this finding. Le Pecq and Paoletti[48] further established that the ethidium cation binds to double-stranded polynucleotides by intercalation between the base planes, with approximately one ethidium binding site per five nucleo-

tides. They have proposed that the enhancement of fluorescence is due to intercalation of the ethidium bromide into the hydrophobic region of the nucleic acids where it is protected against quenching of the fluorescence caused by the aqueous solvent. In support of this proposal is the finding that the fluorescence of ethidium bromide increased when measured in alcohols of increasing hydrophobic character (ranging from ethylene glycol to octanol).

Morgan and Paetkau[49] have found that at an ethidium bromide concentration of 0.5 μg/ml there is a linear response of fluorescence enhancement with double-stranded DNA concentrations up to 0.02 A_{260} per ml. This observation has led to a rapid and convenient assay for the detection and determination of the relative amount of covalently linked complementary DNA (CLC-DNA).[49,50]

Using this assay technique, aliquots of samples containing cross-linked DNA are analyzed for CLC sequences by diluting them in a solution of ethidium bromide buffered to pH 11.6. A measurement of the fluorescence of this solution at 600 nm, with excitation at 525 nm, gives an estimate of the total DNA concentration. The solution is then heat denatured, quickly cooled, and equilibrated to ambient temperature, when the fluorescence of the solution is again measured. Under these conditions, separable strands of DNA do not reanneal. However in CLC sequences the cross-link serves as a nucleation point, allowing rapid renaturation and the concomitant enhance-

ment of ethidium fluorescence. The ratio of the after-heating fluorescence to the before-heating fluorescence is thus a measure of the proportion of covalent cross-linking. The pH of 11.6 is chosen to prevent the spontaneous formation of intrastrand bihelical structures from the separated single strands of DNA formed during the heating-cooling process. These intrastrand structures arise from the presence of short regions of self-complementarity within the DNA strands.[49, 50a] The assay is illustrated in Figure 23.

A modification of this assay using covalently closed circular DNA (CCC-DNA) was employed to study the phenomena of single strand cleavage and alkylation of DNA. With this type of DNA, the uptake of ethidium bromide is restricted by topological constraints. However if the DNA is cleaved in one or more places there is a release of this constraint in going to the open circular (OC) form of DNA, with the result that more ethidium can intercalate, causing increased fluorescence. For PM2 DNA this increase in the before-heating fluorescence amounts to about 30% per nick.[50]

After heat denaturating at pH 11.6 CCC-DNA will completely reanneal, giving a 100% return of fluorescence. With OC-DNA on the other hand, the denaturation causes separation of the linear strand from the circular strand, with the result being that the value of the after-heating fluorescence falls to zero. These processes are illustrated in Figure 24(a) and (b).

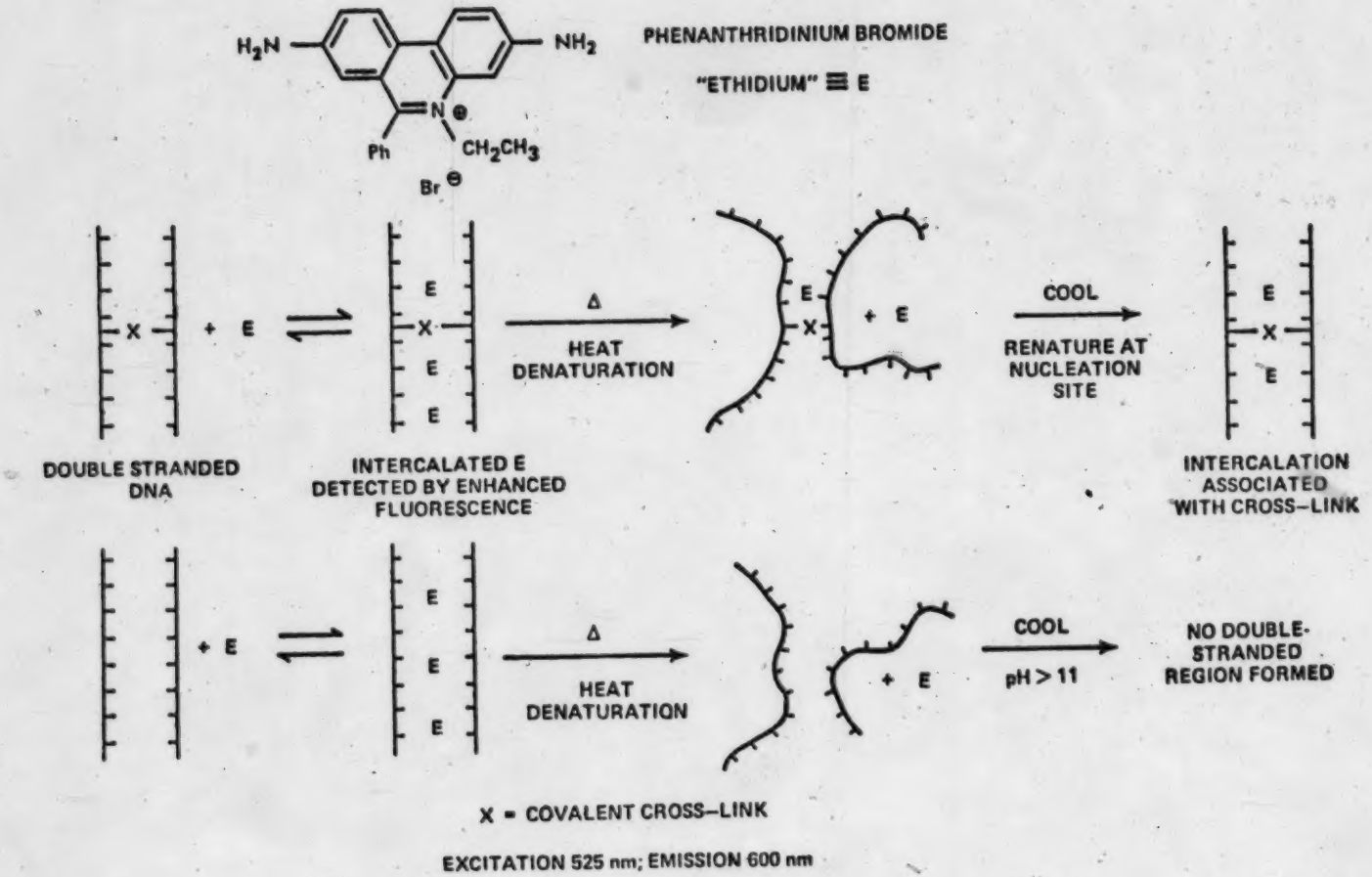


Figure 23. Fluorescence assay for detecting covalent cross-linking of DNA.

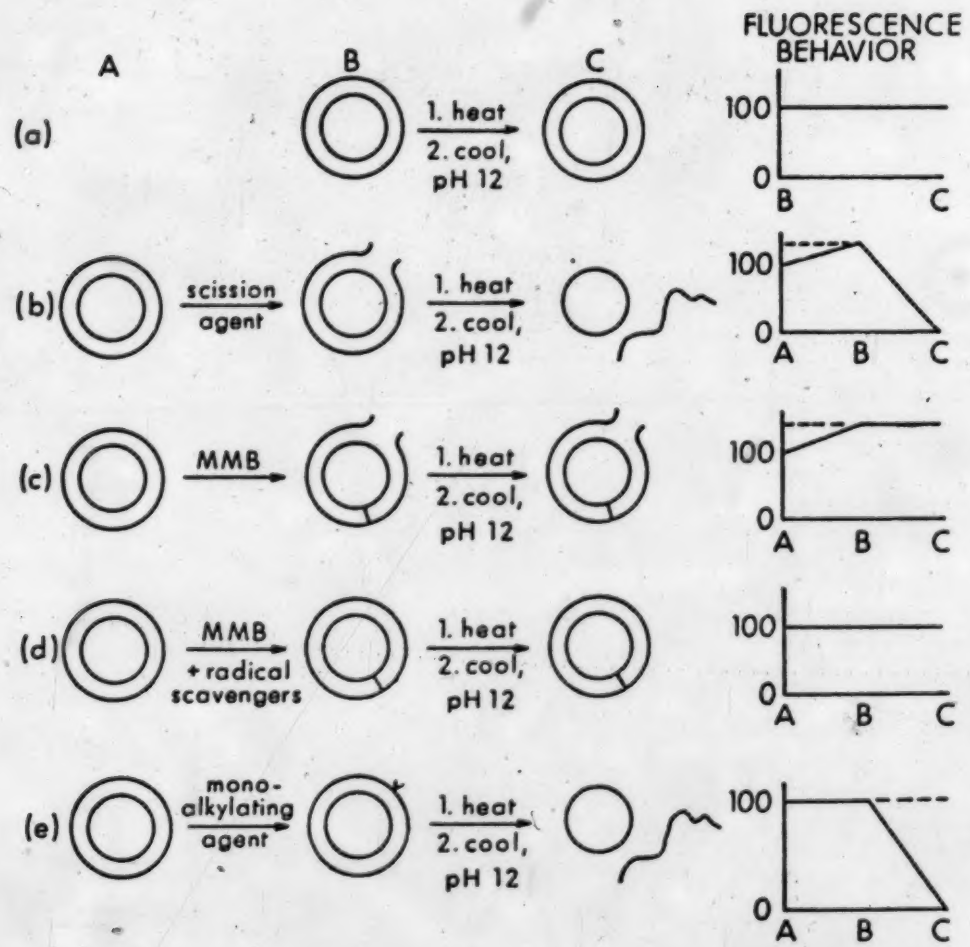


Figure 24. Fluorescence assay for detecting single strand scission, cross-linking and alkylation of CCC-DNA.

Cross-linking or alkylation will complicate this scission assay. If there is cross-linking in addition to scission, the cross-link will serve as a nucleation site, causing full return of fluorescence after heating (Figure 24 (c)). Nevertheless, the rise in the before-heating fluorescence remains characteristic of the scission process. Cross-linking without scission gives only the full return of fluorescence (Figure 24(d)). If there is alkylation in addition to scission, there will still be an increase in the before-heating fluorescence due to the scission process; however in this case the value of the after-heating fluorescence falls to zero, provided there is no accompanying cross-linking. This is caused by a heat-induced depurination in which the alkylated base is expelled from the strand of DNA. The opened strand will separate from the circular strand, causing a drop in fluorescence. Alkylation without strand scission gives a fluorescence after heating which drops to zero, but no rise in the fluorescence prior to heating (Figure 24(e)).

Studies on the Covalent Interaction of Mitomycin B with DNA

Detection of Covalent Cross-linking of DNA by Mitomycin B by the Ethidium Fluorescence Assay

Covalent cross-linking of λ-phage DNA was observed on incubating the DNA with mitomycin B at ambient temperature and pH 7.0. Sodium borohydride was used to reduce the mitomycin B. Timed aliquots were removed and the extent of covalent cross-linking was determined by the ethidium fluor-

escence assay. It was found that mitomycin B produced rapid cross-linking, with over half of the DNA being cross-linked within five minutes. The before-heating fluorescence did not change with time during both this experiment and an analogous experiment in which the assay solution was buffered to pH 7.0, thus suggesting that the alkylation of DNA does not occur at the N-7 position of guanine.[35]

The pH dependence of the cross-linking was established by repeating the original experiment at reaction pH's of 5.0, 6.0, 8.7, and 10.3. The results are given in Table IX and Figure 25.

In contrast to the behavior of streptonigrin 3,[51] it was found that NADH and NADPH did not reduce mitomycin B.

pKa of Mitomycin B

The pKa of mitomycin B in water was determined from the pH of a solution of mitomycin B titrated half way to its equivalence point with dilute hydrochloric acid. This pKa value was found to be 4.3. The pKa of mitomycin C in 50% aqueous methanol is, in contrast, 3.2.[22a]

Dependence of the Efficiency of Covalent Cross-Linking of DNA by Mitomycin B on the (G+C) Content of the DNA

Iyer and Szybalski[19b] and more recently Lown *et al.*[12] have shown that the extent of covalent cross-linking of DNA by mitomycin C increases with increasing (G+C) content of the DNA. We have determined by the ethidium fluorescence assay that this also obtains for mitomycin B. Three natural DNA's of different (G+C) content were used:

Table IX

Per Cent Cross-Linking of λ DNA by Mitomycin B *vs* pH

Conc. MMB x 10^{-4} M	Conc. $NaBH_4$ x 10^{-3} M	pH	% Cross-Linking/ Time to Max. (min)
0.6	1.2	5.0	100/5
		6.0	89/5
		7.0	45/5
		8.7	32/5
		10.3	0/5
1.0	2.0	6.0	100/5
		7.0	69/5
		8.7	27/5
		10.3	0/5
2.0	3.6	6.0	95/5
		7.0	67/5
		8.7	29/5
		10.3	0/30
4.0	5.6	5.0	100/5
		6.0	92/5
		7.0	68/5
		8.7	35/5
		10.3	0/30

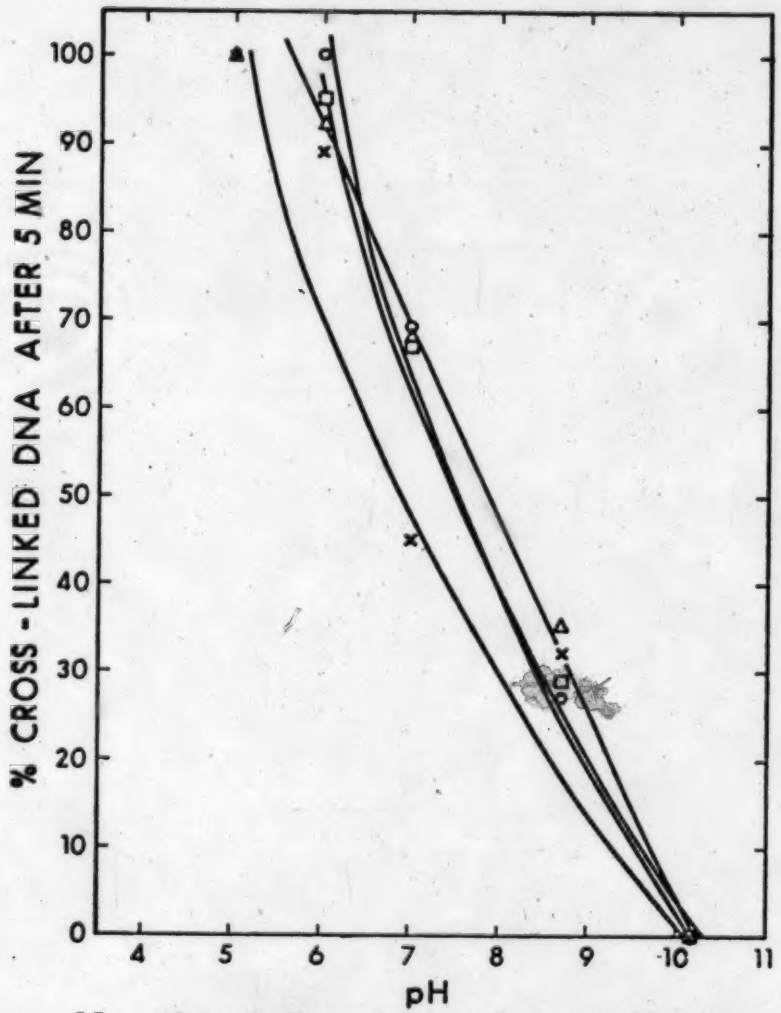


Figure 25. The pH dependence of cross-linking of DNA by reduced mitomycin B after 5 min. The reactions were at 23° in 50 mM buffer at the appropriate pH and λ DNA at 0.9 A_{260}. The mitomycin B concentrations were 0.6, 1.0, 2.0, and 4.0×10^{-4} M and the sodium borohydride 1.2, 2.0, 3.6, and 5.6×10^{-3} M for the following symbols respectively: X-X, O-O, □-□, △-△. Fluorescence values were obtained after heating.

Clostridium perfringens (30%), calf thymus (40%), and *Escherichia coli* (50%). The results for the three DNA's obtained by the assay method are not strictly comparable since the DNA's have different molecular weights, as determined by sedimentation velocities (Table X). One

Table X

Average Molecular Weights of DNA's Determined by Sedimentation Velocities

DNA	Avg. Mol. Wt.
C. perfringens	11.4×10^6
Calf thymus	9.1×10^6
E. coli	7.7×10^5

cross-link per DNA molecule is sufficient to produce rapid reannealing of the DNA after cooling, regardless of the length of the strand. As a result, DNA's of lower molecular weight will require a greater number of cross-linking events on a per nucleotide basis to obtain the same overall per cent cross-linking. Assuming a Poisson's distribution of cross-links and that one cross-link will permit renaturation of the molecule of which it is a part, an estimate of the average number of cross-links per molecule (m) was made from $m = \ln (1/P_o)$, where P_o is the fraction of molecules not cross-linked. Taking the average molecular weight for each nucleotide as 300, the number of cross-links per

nucleotide was calculated for each of the DNA's. These results are tabulated in Table XI. Figure 26 shows that there is a direct correlation between the extent of cross-linking and the per cent (G+C) content of the DNA.

Cross-Linking by Mitomycin B Without Reduction

Iyer and Szybalski[19b] have proposed that the C-1 and C-10 sites of mitomycin C are activated by reduction of the quinone moiety followed by the elimination of methanol (Scheme 3). It has been found,[12] however, that at sufficiently low pH, cross-linking occurs even in the absence of reduction. We have found that this is also the case for mitomycin B. Figure 27 shows the kinetics of the cross-linking of DNA by unreduced mitomycin B at pH 4.0. The extent of cross-linking is greater than for mitomycin C[12] as is the initial rate of cross-linking. These findings are consistent with the higher pKa and the greater pH dependence of cross-linking exhibited by mitomycin B. A control experiment indicated that under these conditions there was no significant acid-induced cross-linking of the DNA. A similar experiment at pH 5.0 showed no cross-linking after sixty minutes.

Detection of Alkylation of DNA by Mitomycin B

It has been found[13] that as the concentration of mitomycin C in the reaction mixture is increased, a progressive decrease in the fluorescence is observed. Subsequent experiments established that this phenomenon was due to a reduction of the number of ethidium bromide

Table XI

Dependence of Efficiency of Covalent Cross-Linking by Mitomycin B on the (G+C) Content of the DNA

MMB Conc. x 10^{-4} M	$NaBH_4$ Conc. x 10^{-3} M	% (G+C)	Max. % Cross-Linking/Time (min)	Avg. Cross-Links per Molecule	Avg. Cross-Links per Nucleotide x 10^{5}
0.6	1.2	30	8/10	0.083	0.22
0.6	1.2	40	23/10	0.261	0.86
0.6	1.2	50	16/10	0.174	6.79
3.0	6.2	30	14/20	0.151	0.40
3.0	6.2	40	23/10	0.357	1.18
3.0	6.2	50	30/10	0.261	10.2

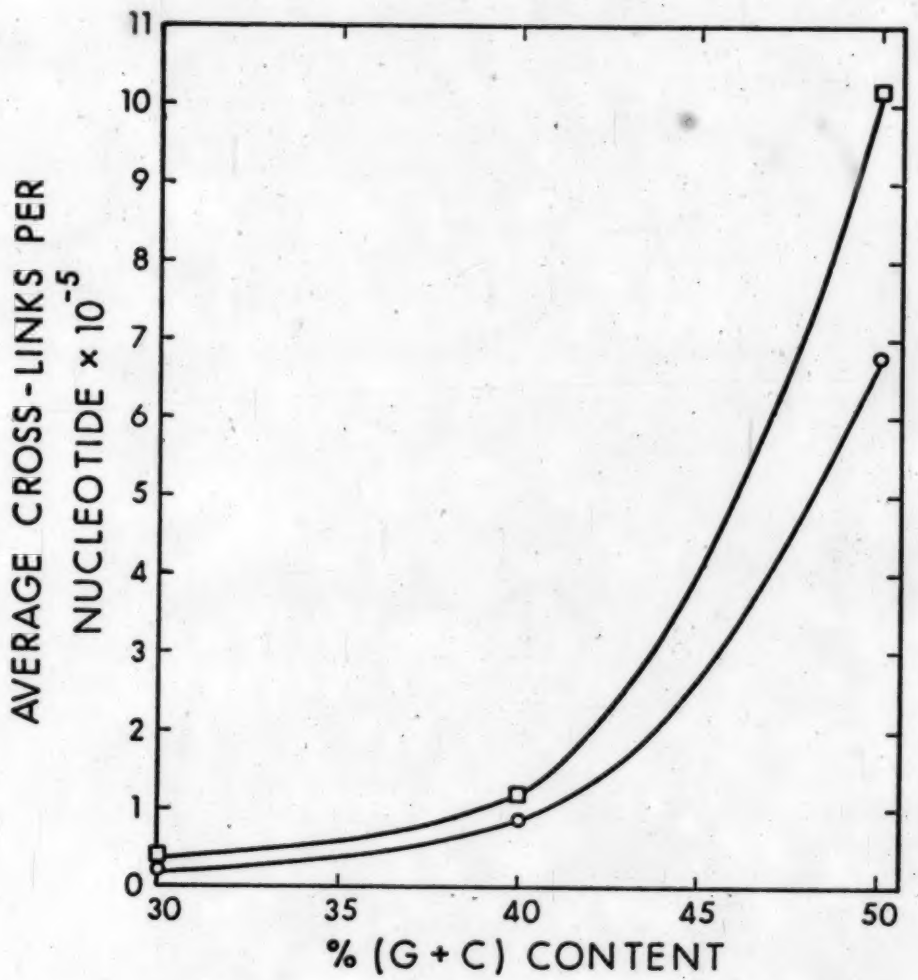


Figure 26. Dependence of efficiency of covalent cross-linking of DNA's by reduced mitomycin B on the (G+C) content of the DNA. Reactions contained DNA at 1.25 A_{260}, 0.05 M phosphate buffer pH 7.0, and mitomycin B and sodium borohydride at ○ 0.6×10^{-4} M, 1.2×10^{-3} M and □ 3.0×10^{-4} M, 6.2×10^{-3} M, respectively.

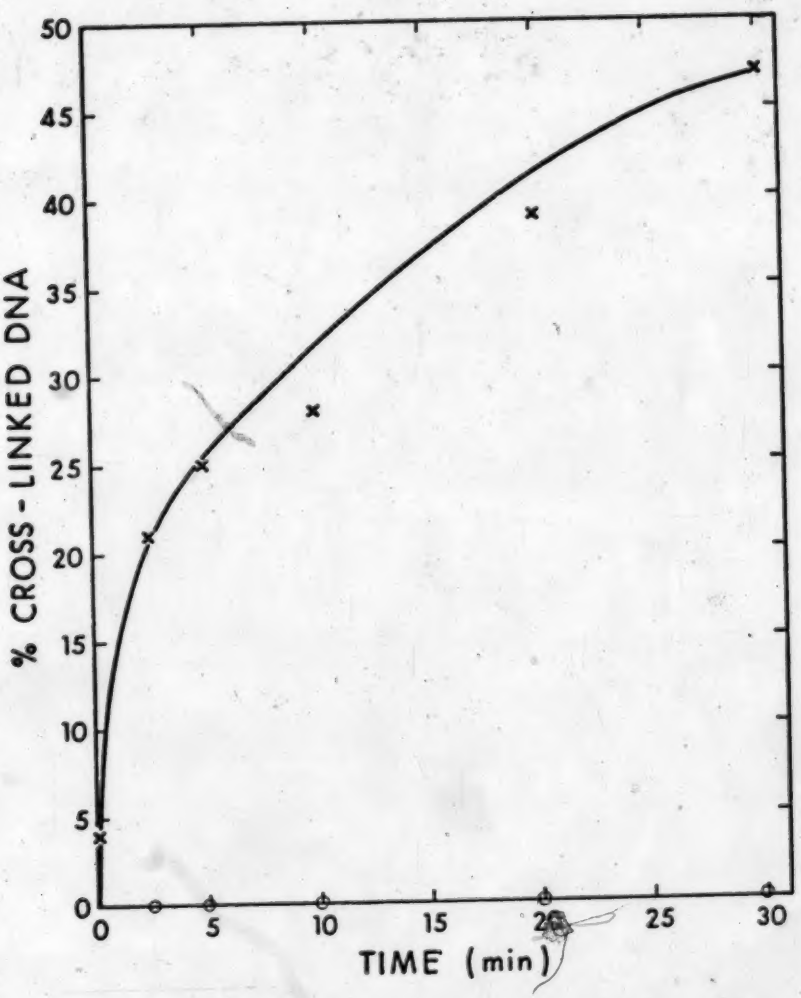


Figure 27. The cross-linking of λ DNA by mitomycin B without reduction. The reaction mixture contained λ DNA at 0.95 A_{260}, 50 mM sodium acetate buffer pH 4.0 and mitomycin B at 2.4×10^{-4} M, at 23°. 10 µl aliquots were added to the ethidium assay solution and the per cent cross-linked DNA was equated to the per cent fluorescence remaining after heating. ○ Control experiment at pH 4.0 with no mitomycin added.

intercalation sites by either steric hindrance or charge repulsion between the positively charged alkylated bases and the ethidium cation. We have observed a similar decrease in fluorescence with increasing concentrations of mitomycin B. These results are presented in Table XII and Figure 28, together with their pH dependence.

Correlation of the Decrease in Fluorescence With the Extent of Binding of Mitomycin B to DNA

To confirm that the loss of fluorescence observed during the ethidium assay is due to alkylation of the DNA, an experiment was performed to correlate this decrease in fluorescence with the extent of the binding of the mitomycin B to the DNA. The decrease in fluorescence with progressively increasing amounts of mitomycin B was determined by the fluorescence assay described above. The binding ratios of the DNA to mitomycin B were determined by the ^{3}H-label method described by Begleiter,[13] except that the concentration of bound antibiotic was obtained from the uv absorbance of the drug-DNA complex at 286 nm. The proximity of this absorption to the DNA absorption at 260 nm with the resulting overlap of absorptions, and the lower mitomycin concentrations used, cause a larger uncertainty in the bound mitomycin B concentrations than was found in the case of the mitomycin C concentrations; this uncertainty becomes particularly large for the smaller concentrations of mitomycin B. As a result it is not possible to establish whether or

Table XII

Per Cent Loss of Fluorescence in Alkylation of λ DNA by Mitomycin B *vs* pH

Conc. MMB $\times 10^{-4}$ M	Conc. $NaBH_4$ $\times 10^{-3}$ M	pH	% Loss of Fluorescence
0.6	1.2	5.0	34
		6.0	12
		7.0	1
		8.7	0
1.0	2.0	5.0	53
		6.0	16
		7.0	2
		8.7	0
2.0	3.6	5.0	69
		6.0	33
		7.0	20
		8.7	0

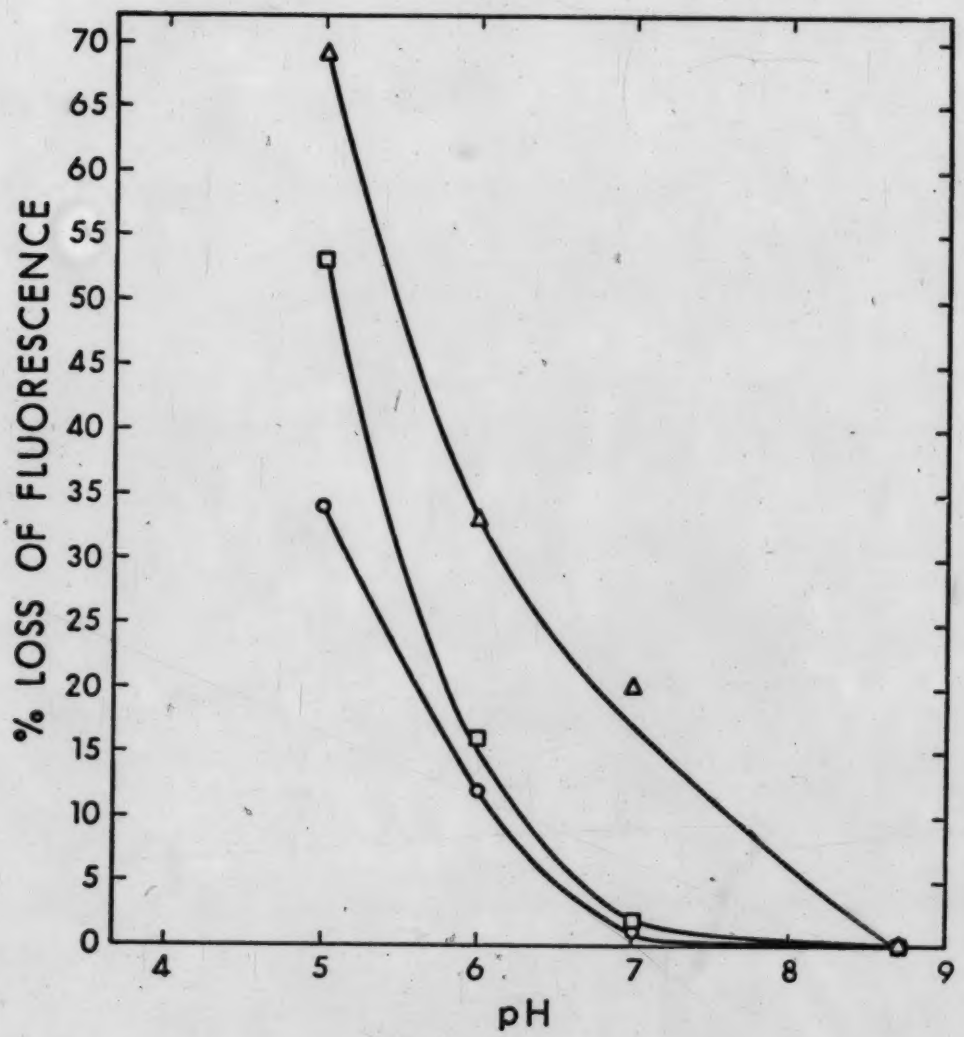


Figure 28. The pH dependence of alkylation of DNA by reduced mitomycin B. The reactions were at 23° in 50 mM buffer at the appropriate pH and λ DNA at 0.9 A_{260}. The mitomycin B concentrations were 0.6, 1.0, and 2.0 x 10^{-4} M and the sodium borohydride 1.2, 2.0 and 3.6 x 10^{-3} M for the following symbols respectively:

○-○, □-□, △-△.

not there is a strong correlation between the loss of fluorescence and the binding ratio; nevertheless the data of Table XIII and Figure 29 show that there is at least a trend in this direction.

The Mechanism of DNA Degradation by Mitomycin B

Detection of Mitomycin B Induced Single Strand Scission of CCC-DNA by the Fluorescence Assay

Since the breakdown of DNA by mitomycin C has been suggested[29] as a possible mode of action for that drug, the interaction of mitomycin B with CCC-DNA was studied in order to gain information about the DNA degradation by this drug, and by the mitomycins in general.

On exposing the CCC-DNA derived from PM2 bacteriophage to mitomycin B reduced *in situ*, a rapid increase in fluorescence was observed. There was no loss of fluorescence observed after heating, which shows that there is also efficient cross-linking of the DNA. The overall reaction is that illustrated in Figure 24(c). The results are shown in Figure 30.

It was also shown in independent experiments that this cleavage of the DNA can be retarded by the addition of either or both of the cell protective enzymes catalase (which removes hydrogen peroxide from the cell) and superoxide dismutase (which catalyzes the dismutation of the superoxide ($O_2^{\ominus\cdot}$) radical). The strand cleavage could also be inhibited by the presence of such free radical scavengers as isopropyl alcohol, dimethyl sulfoxide,

Table XIII

Correlation of Per Cent Loss of Fluorescence with the DNA-Mitomycin B Binding Ratio

Initial MMB Conc. x 10^{-5} M	% Loss of Fluorescence	Nucleotide Conc. After Dialysis x 10^{-4} M	Absorbance of Complex at 286 nm	Bound MMB Conc. x 10^{-6} M	Binding Ratio
0.0	0	1.55	0.087	0.0	-----
1.8	37	1.76	0.461	2.8 ± 1.0	63.7 ± 20
5.5	50	1.74	0.461	3.2 ± 0.5	55.1 ± 9
9.2	76	1.63	0.435	3.1 ± 0.7	51.8 ± 10
18.3	86	1.86	0.532	6.6 ± 0.4	28.0 ± 2
27.5	90	1.54	0.463	7.5 ± 0.2	20.6 ± 1

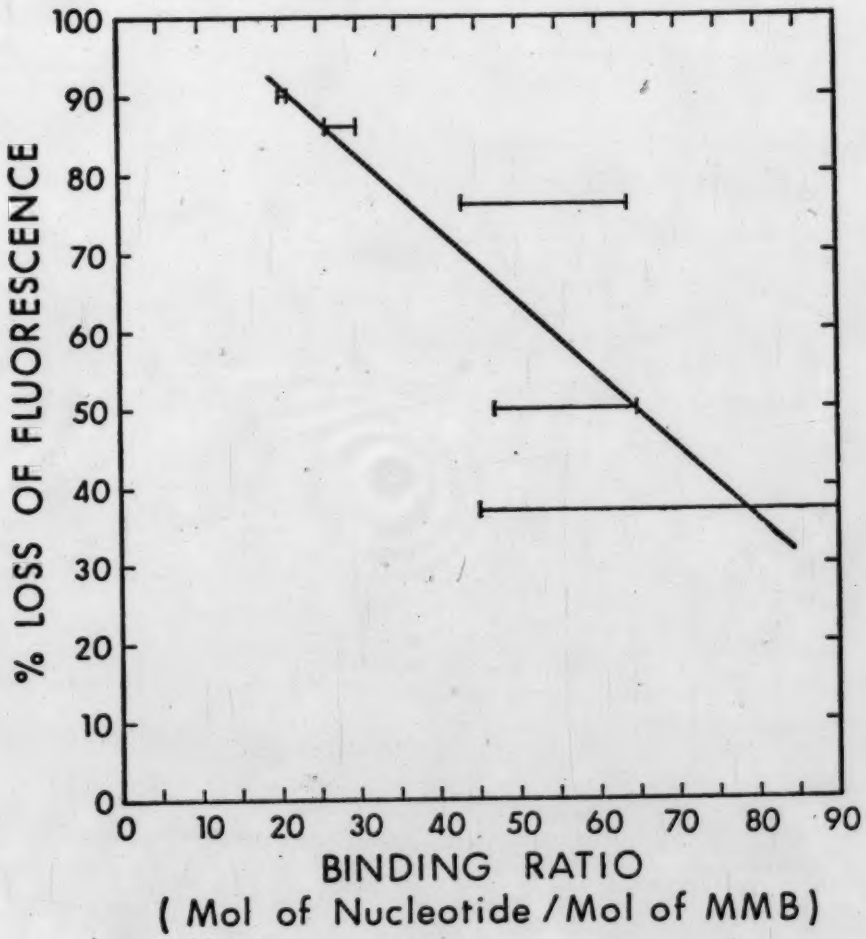


Figure 29. Dependence of per cent loss of fluorescence on the DNA-Mitomycin B binding ratio.

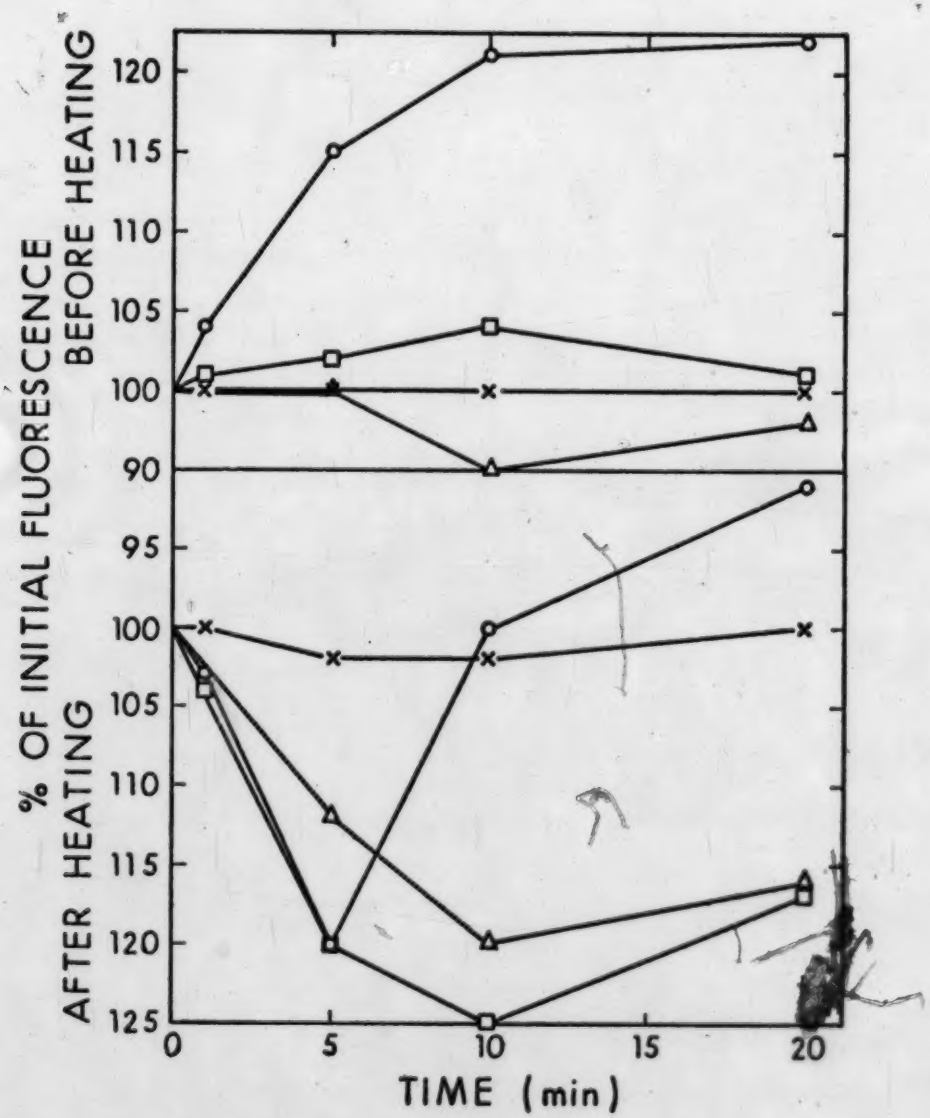


Figure 30.

Single strand scission of PM2 CCC-DNA by mitomycin B. Reactions were performed at ambient temperature in a phosphate buffer pH 7.0 with 0.6 A_{260} of PM2 DNA. (a) Additional components were: ○ mitomycin B 3.3×10^{-4} M, sodium borohydride 5.5×10^{-3} M; □ mitomycin B 3.3×10^{-4} M, sodium borohydride 5.5×10^{-3} M, sodium benzoate 0.05 M; △ mitomycin B 3.3×10^{-4} M, sodium borohydride 4.4×10^{-3} M, potassium iodide 0.05 M; × control.

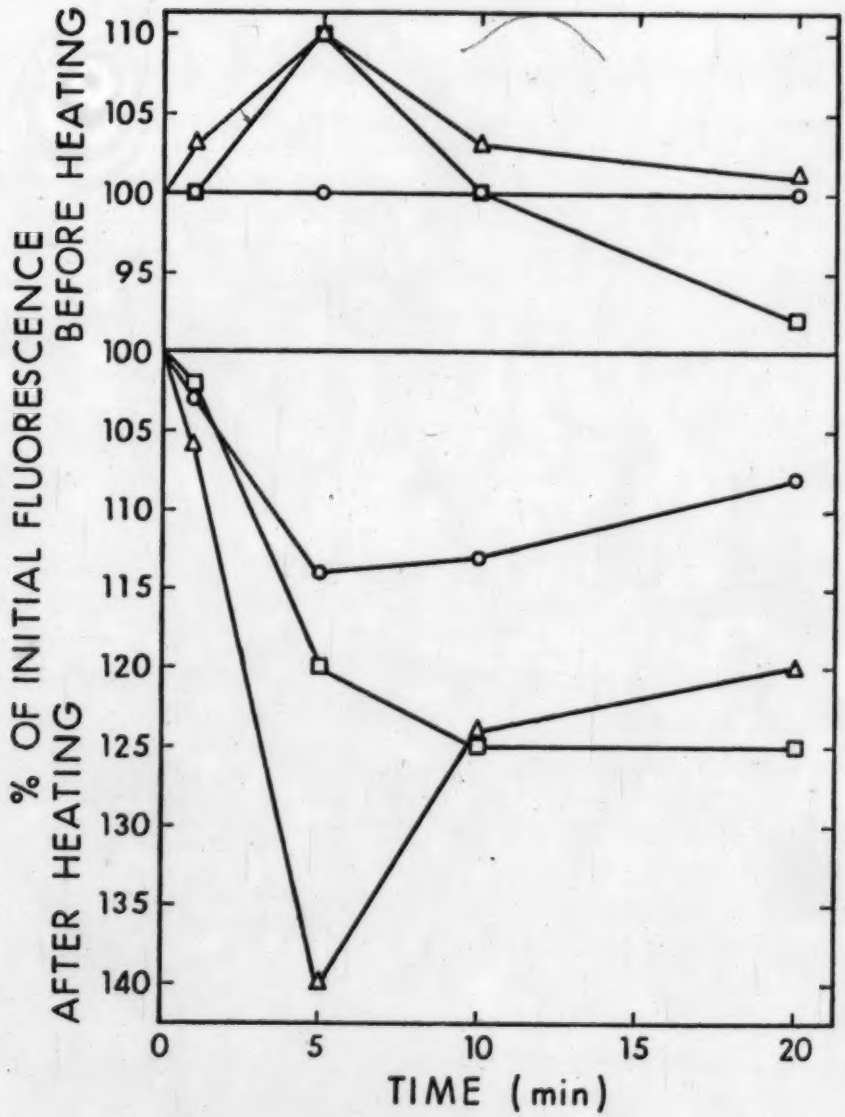


Figure 30 (continued).

(b) Additional components were: ○ mitomycin B 3.3×10^{-4} M, sodium borohydride 5.5×10^{-3} M, isopropyl alcohol 0.25 M; □ mitomycin B 3.5×10^{-4} M, sodium borohydride 5.7×10^{-3} M, dimethyl sulfoxide 0.25 M; △ mitomycin B 3.3×10^{-4} M, sodium borohydride 4.6×10^{-3} M, 2-mercaptoethanol 0.25 M.

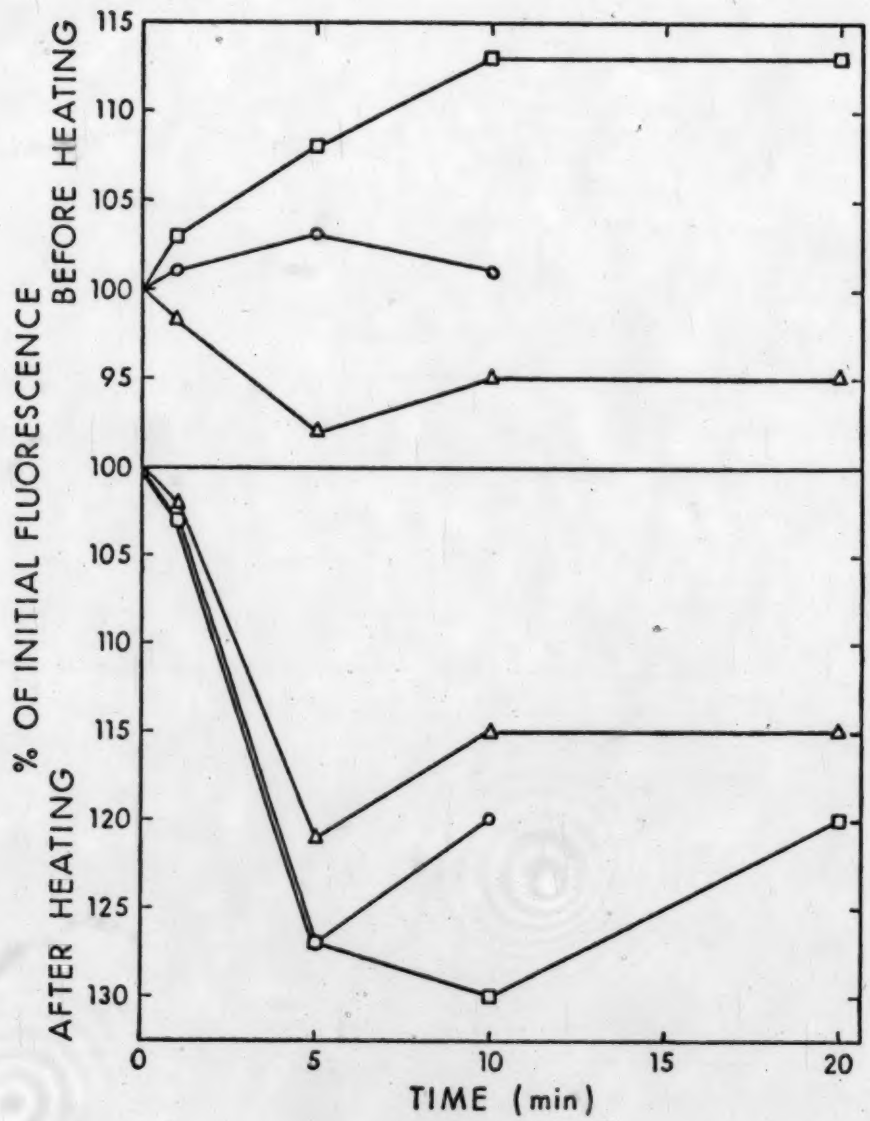


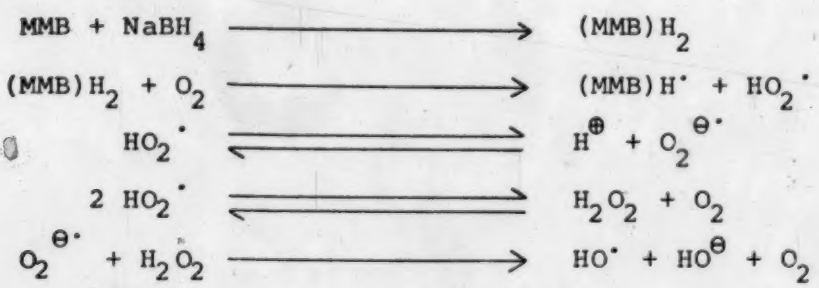
Figure 30 (continued).

(c) Additional components were: ○ mitomycin B 3.3×10^{-4} M, sodium borohydride 5.5×10^{-3} M, catalase 2.3×10^{-7} M; □ mitomycin B 3.3×10^{-4} M, sodium borohydride 5.5×10^{-3} M, superoxide dismutase 1.5×10^{-5} M; △ mitomycin B 3.5×10^{-4} M, sodium borohydride 5.7×10^{-3} M, catalase 2.4×10^{-7} M, superoxide dismutase 1.5×10^{-5} M.

2-mercaptoethanol, sodium benzoate, and potassium iodide (Figure 30).

These results suggest a mechanism similar to that proposed for mitomycin C[12] and streptonigrin,[26] in which the cleavage of the DNA is induced by hydroxyl radicals (Scheme 15).

Scheme 15



Consistent with this type of mechanism for mitomycin C are the recent findings of Handa and Sato[52] who confirmed that mitomycin C facilitates the production of the superoxide radical on reduction, and Tomasz[53] who found that reduced mitomycin C generates hydrogen peroxide on exposure to air.

Strand Scission of PM2 CCC-DNA by Covalently Bound Mitomycins

We performed experiments on radical-induced scission

in which either mitomycin B or mitomycin C was covalently attached to DNA at pH 4.0, without reduction. Unattached mitomycins were removed by dialysis, and the DNA-mitomycin complexes were then reduced by sodium borohydride, causing the characteristic rise in before-heating fluorescence. Control experiments showed that all unattached mitomycins had been removed. The cleavage produced by mitomycin C was found to be inhibited by isopropyl alcohol, superoxide dismutase, and, in contrast to previously reported findings,[13] by catalase. In the case of mitomycin B, all three inhibitors reduced the rise in fluorescence; this was especially true for the two enzymes. In both cases there was a progressive rise in the after-heating fluorescence due to the formation of further cross-links after reduction (Figures 31 and 32). We conclude that DNA may be cleaved by mitomycins to which it is covalently attached, as well as by the free antibiotic.

Effect of Intercalated Ethidium on the Mitomycin B and Mitomycin C Induced Single Strand Scission and Cross-Linking of PM2 DNA

It was of interest to see what effect the presence of ethidium bromide in the primary binding sites of DNA would have on the radical-induced scission of DNA.[54] Accordingly, PM2 DNA was first equilibrated with varying amounts of ethidium bromide, then treated with the appropriate mitomycin, reduced, and assayed in the usual manner. The solutions were stoppered and protected from

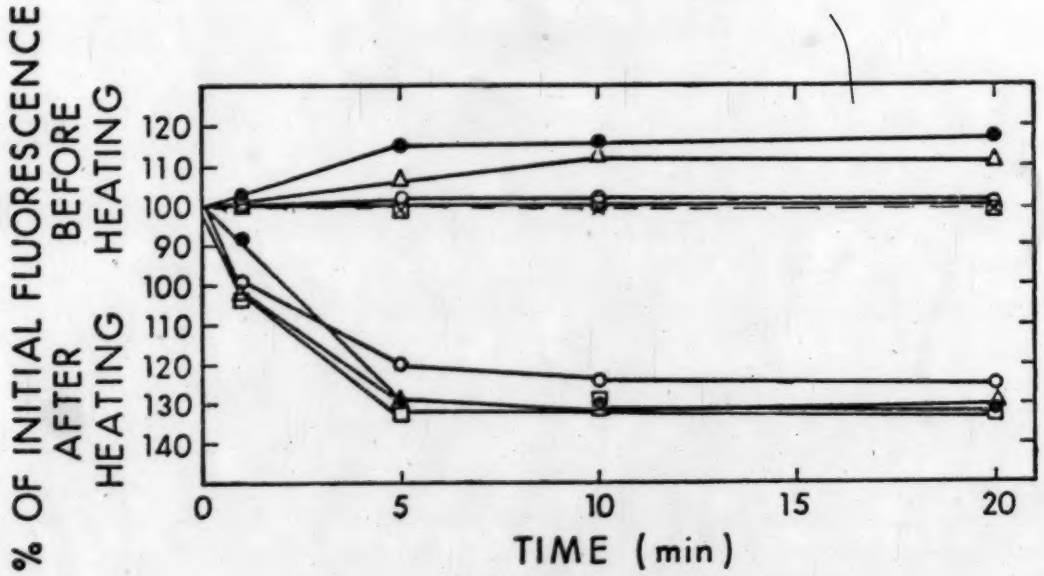


Figure 31. Single strand scission of mitomycin B alkylated PM2 CCC-DNA. PM2 DNA was alkylated at ambient temperature in 0.05 M sodium acetate buffer pH 4.0 containing 2.4 A_{260} of PM2 DNA and 5.0×10^{-4} M mitomycin B. Dialysis afforded alkylated DNA. Scission reactions were performed at ambient temperature in phosphate buffer pH 7.0 containing 1.08 A_{260} of alkylated DNA. Additional components were: ● sodium borohydride 6.1×10^{-3} M; □ sodium borohydride 6.1×10^{-3} M, catalase 2.4×10^{-7} M; ○ sodium borohydride 6.1×10^{-3} M, superoxide dismutase 1.5×10^{-5} M; △ sodium borohydride 6.1×10^{-3} M, isopropyl alcohol 0.25 M; × control.

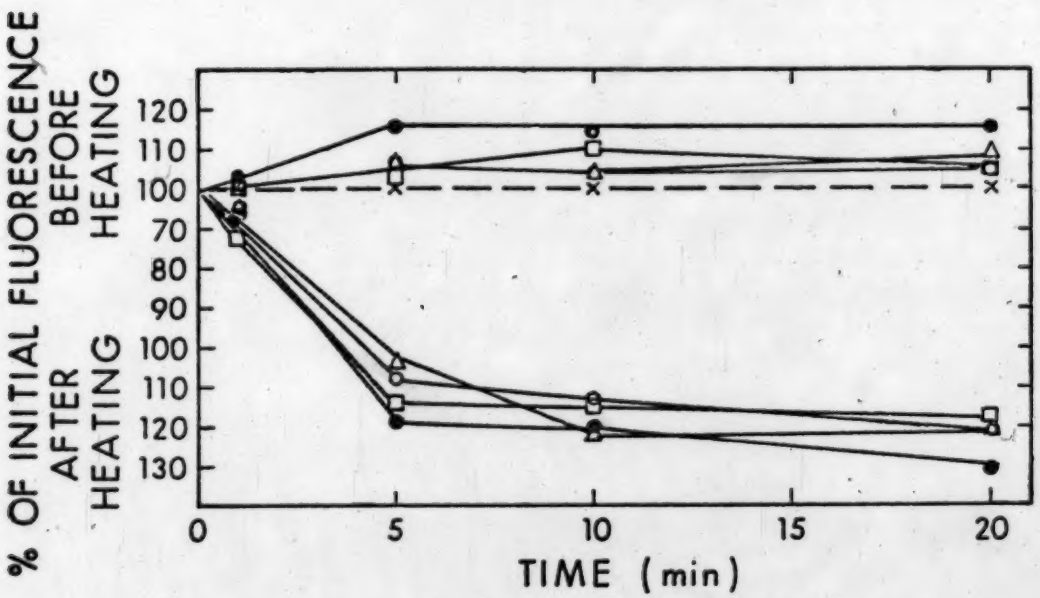


Figure 32. Single strand scission of mitomycin C alkylated PM2 CCC-DNA. PM2 DNA was alkylated at ambient temperature in 0.05 M sodium acetate buffer pH 4.0 containing 2.4 A_{260} of PM2 DNA and 5.7×10^{-4} M mitomycin C. Dialysis afforded alkylated DNA. Scission reactions were performed at ambient temperature in phosphate buffer pH 7.0 containing 1.08 A_{260} of alkylated DNA. Additional components were : ● sodium borohydride 5.9×10^{-3} M; □ sodium borohydride 5.9×10^{-3} M, catalase 2.4×10^{-7} M; ○ sodium borohydride 5.9×10^{-3} M, superoxide dismutase 1.5×10^{-5} M; △ sodium borohydride 5.9×10^{-3} M, isopropyl alcohol 0.25 M; X control.

light to prevent cleavage of the DNA due to the ethidium bromide.

For the case of mitomycin B it was found that with no added ethidium bromide there was a rise in the before-heating fluorescence, characteristic of strand scission. The prior addition of 6.3×10^{-6} M, 2.5×10^{-5} M, or 6.3×10^{-5} M ethidium bromide caused no increase in this rise (Figure 33). These results indicate that the presence of intercalated ethidium does not affect the strand scission caused by mitomycin B. The after-heating fluorescence with no added ethidium bromide showed an initial rise, characteristic of cross-linking of the open circular (OC) DNA present in the CCC-DNA, followed by a later drop, characteristic of strand scission. With added ethidium there was only the drop of after-heating fluorescence observed, with this drop becoming more rapid with increasing concentration of ethidium bromide, until the saturation level is reached (Figure 33). This confirms that there is interference with the cross-linking process.

In the case of mitomycin C it was observed that with no added ethidium bromide there was an increase in the before-heating fluorescence, characteristic of strand scission. With the prior addition of ethidium bromide in concentrations of 6.3×10^{-6} M, 2.5×10^{-5} M, or 6.3×10^{-5} M there was an increase in this rise (Figure 34), suggesting that the ethidium bromide is interfering

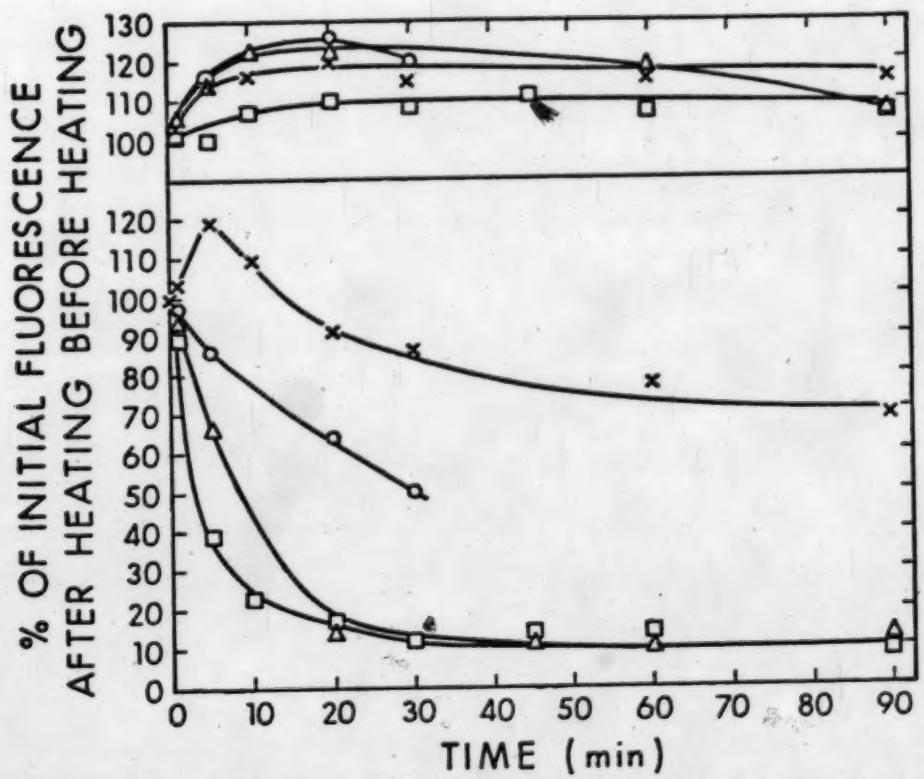


Figure 33. Changes in fluorescence produced by mitomycin B in PM2 CCC-DNA treated with ethidium bromide. The DNA was equilibrated with ethidium bromide at ambient temperature in 0.05 M phosphate buffer pH 7.0 containing 0.6 A_{260} of PM2 DNA. Mitomycin B 1.0×10^{-4} M and sodium borohydride were added and the fluorescence was determined by the ethidium bromide assay. Additional components were : × sodium borohydride 2.5×10^{-3} M; ○ sodium borohydride 2.2×10^{-3} M, ethidium bromide 6.3×10^{-6} M; □ sodium borohydride 2.2×10^{-3} M, ethidium bromide 2.5×10^{-5} M; △ sodium borohydride 2.9×10^{-3} M, ethidium bromide 6.3×10^{-5} M.

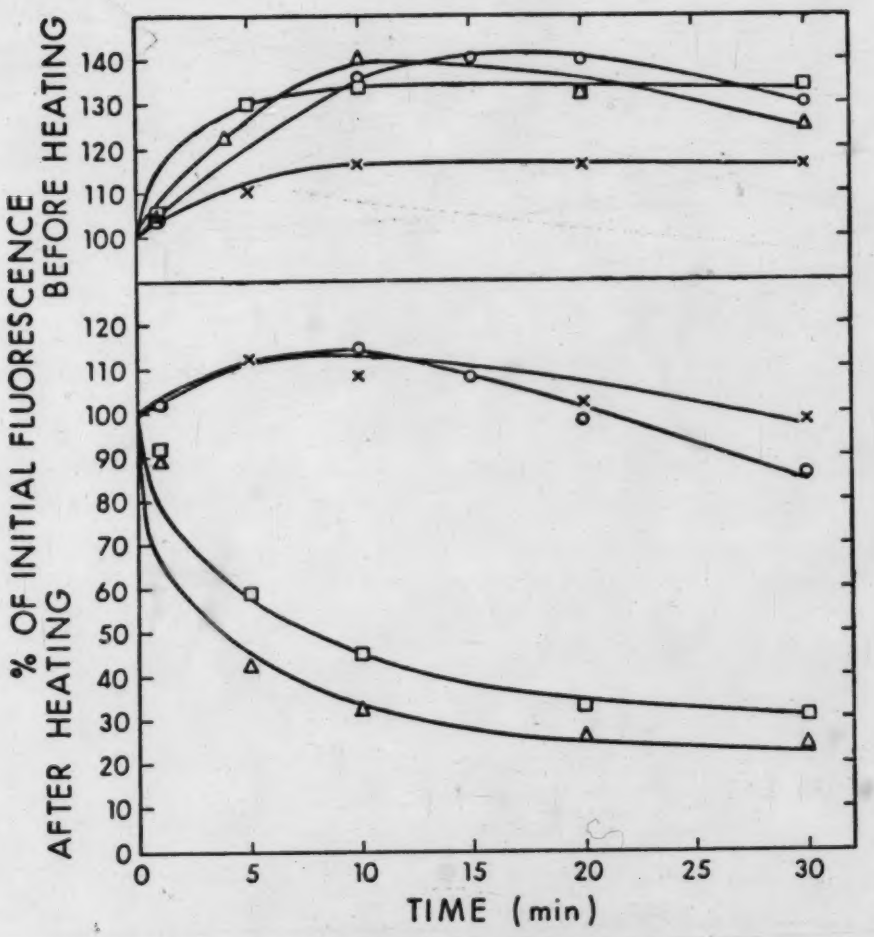
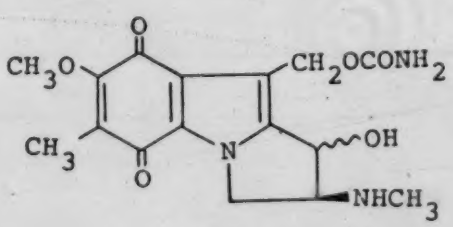


Figure 34. Changes in fluorescence produced by mitomycin C in PM2 CCC-DNA treated with ethidium bromide. The DNA was equilibrated with ethidium bromide at ambient temperature in 0.05 M phosphate buffer pH 7.0 containing 0.6 A_{260} of PM2 DNA. Mitomycin C 1.5 x 10^{-4} M and sodium borohydride were added and the fluorescence was determined by the ethidium bromide assay. Additional components were:. × sodium borohydride 3.5 x 10^{-3} M; ○ sodium borohydride 3.3 x 10^{-3} M, ethidium bromide 6.3 x 10^{-6} M; □ sodium borohydride 3.3 x 10^{-3} M, ethidium bromide 2.5 x 10^{-5} M; △ sodium borohydride 3.4 x 10^{-3} M, ethidium bromide 6.3 x 10^{-5} M.

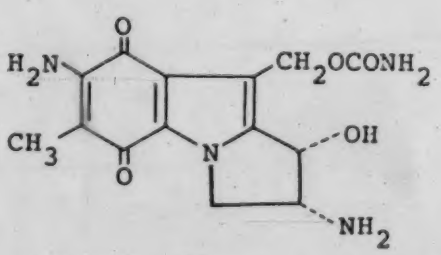
with the other processes such as cross-linking, thereby allowing more of the mitomycin C to engage in strand scission. The after-heating fluorescence rose then fell when there was no added ethidium; this was unchanged when the added ethidium concentration was 6.3×10^{-6} M. However at the higher ethidium concentrations there was an immediate drop in the after-heating fluorescence. This confirms that there is interference with the cross-linking at higher ethidium concentrations.

Studies on Aziridine-Ring-Opened Mitomycin B

On treatment with dilute acid, mitomycin B forms the ring-opened derivative 55. As for the corresponding derivative of mitomycin C, 56, this compound showed no evidence of cross-linking DNA upon reduction; however it



55



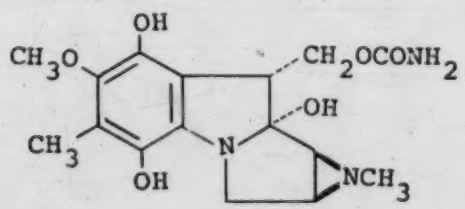
56

did cause rapid strand scission. This scission could be suppressed by the addition of isopropyl alcohol as a radical scavenger. With the scission inhibited in this manner, alkylation of DNA was observed. This alkylation proceeded

more slowly than with the corresponding mitomycin C derivative, causing a fluorescence loss of 8% in 20 min and 24% in 1 hr (Figure 35). The mitomycin C derivative lowered fluorescence 24% in 20 min and 59% in 3 hr.[13]

Electrochemical Studies of Mitomycin B and its Aziridine-Ring-Opened Derivative

Mitomycin B and its aziridine-ring-opened derivative 55 were investigated by HMDE cyclic voltammetry in aqueous media at 37.5°. It was found that the initial reduction of mitomycin B is a reversible two-electron, two-proton reduction of the quinone moiety to the corresponding hydroquinone 57, occurring at a potential of -0.200 ± 0.005 V (Process I). The derivative 55 also



57

undergoes a reversible two-electron, two-proton reduction of the quinone moiety, at a potential of -0.320 ± 0.005 V (Process II) (Scheme 16).

In addition, three other electrochemical processes were observed, two of which were irreversible, the other

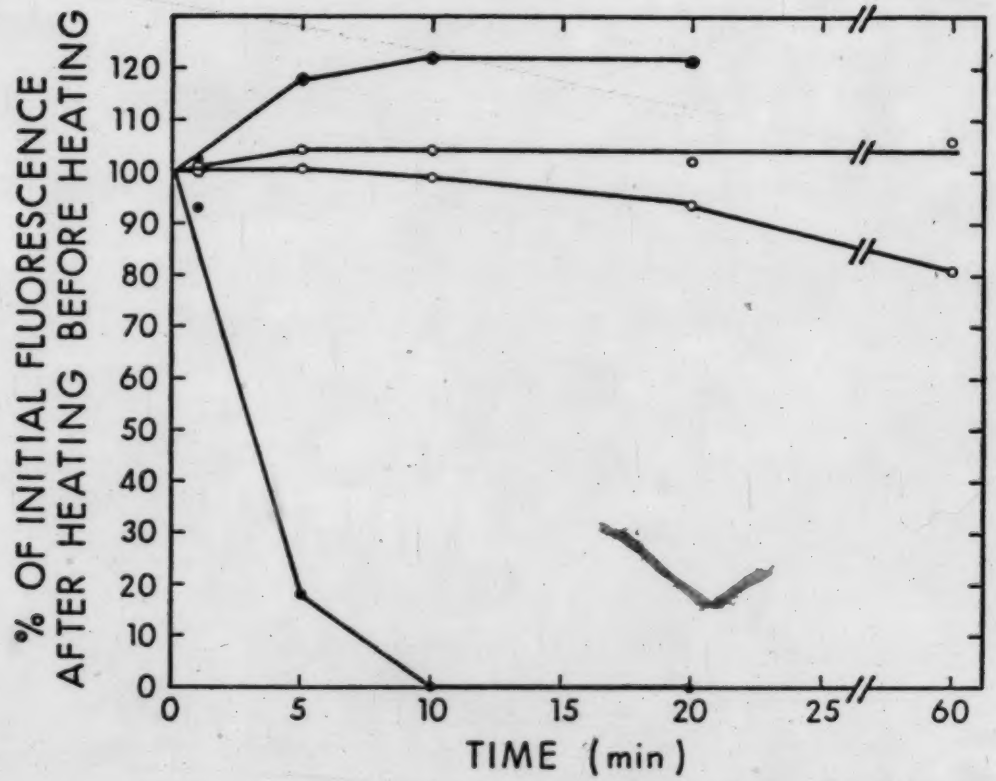
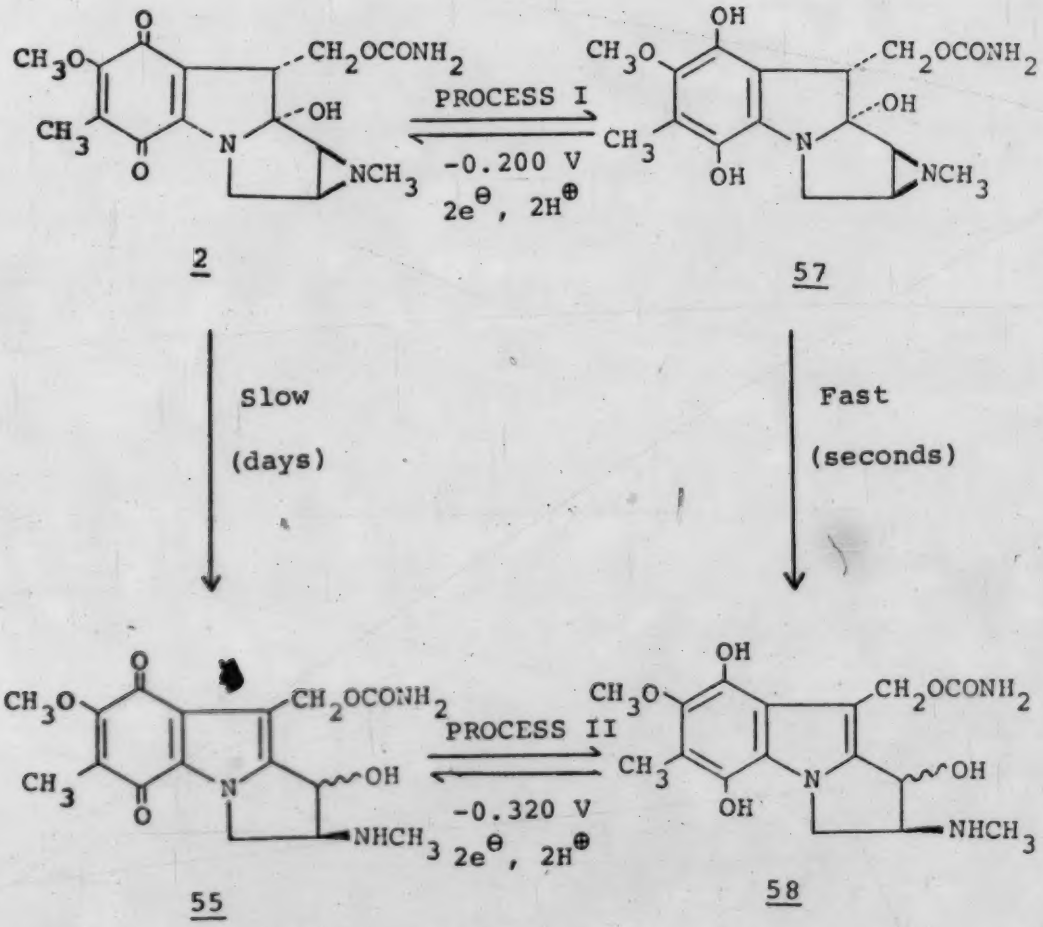


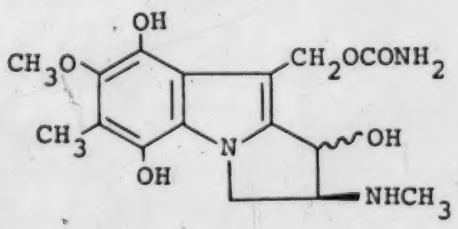
Figure 35. Single strand scission and alkylation of PM2 CCC-DNA by the mitomycin B aziridine-ring-opened derivative 55. Reactions were performed at ambient temperature and contained PM2 DNA at 0.6 A_{260}, 0.05 M phosphate buffer pH 7.0 and sodium borohydride 4.9×10^{-3} M. Other components were: ● aziridine-ring-opened compound 55 3.3×10^{-4} M; ○ compound 55 3.3×10^{-4} M, isopropyl alcohol 0.25 M.

Scheme 16



2 57 55 58

being possibly reversible; however these processes have not been unambiguously defined other than that they appear to be due to further reduction products of the hydroquinone 58. They do not correspond to loss of the carbamate



58

moiety since soluble carbamates were found to be electrochemically inactive over the potential range studied.

There was no evidence of peak splitting such as would be expected if there were a semiquinone intermediate with a lifetime greater than about 1 msec present in the reduction of mitomycin B. However these measurements were performed under a nitrogen atmosphere, and so the results do not exclude the possibility of the formation of semiquinone intermediates *in vivo* or by air oxidation of the corresponding hydroquinones in an oxygenated solution. The semiquinone of mitomycin B has been detected upon reduction of the antibiotic in dimethylformamide.[23]

Summary and Conclusions

Mitomycin B differs from mitomycin C in the stereochemistry of three centers and in the nature of three substituents on the mitosane skeleton, though in the activated form only two of these are present. The change from the 7-amino group of mitomycin C to the 7-methoxy group of mitomycin B will be reflected in a change of the electrochemical potential of the quinone and chemical behavior related to this, while the methyl substituent on the aziridine ring of mitomycin B will influence the basicity of the antibiotic. These effects have been borne out by our experimental findings.

The larger pKa value found for mitomycin B compared to that for mitomycin C,[22a] together with the greater pH dependence of cross-linking and the faster and more extensive cross-linking at low pH in the absence of reduction support the suggestion[12] that the aziridine moiety is involved in the initial alkylation of DNA. That the extent of cross-linking for both mitomycins shows a (G+C) dependence and that the extents of alkylation are neither time nor assay-pH dependent for both mitomycins suggest that they bind at the same sites of DNA, possibly the O-6 or N-3 positions of two adjacent guanosines.[35]

It has been suggested that the second position of the mitomycin C molecule involved in cross-linking may be the 7-amino group, and that the binding observed with the

aziridine-ring-opened derivative 56 may be due to reaction at this site rather than at the C-10 position. For mitomycin B, which lacks the 7-amino substituent, reaction at this site is not possible, and so the alkylation observed with the derivative 55 offers firm evidence that the C-10 position does serve as a site for covalent attachment. However, since the rate of alkylation is significantly slower for mitomycin B than for mitomycin C under these conditions, the possibility of a contribution from the 7-amino substituent cannot be excluded, although the difference in rates may be due to inherent differences in the reactivities of the two mitomycins caused by their stereochemical configurations. Studies on another mitomycin such as mitomycin A would shed further light on this matter, since mitomycin A has the same geometry as mitomycin C, but lacks the 7-amino substituent.

The potential of the quinone reduction of mitomycin B is -0.200 V as compared to a value of -0.368 V for mitomycin C,[55] thus indicating that mitomycin B is easier to reduce than mitomycin C, but that the corresponding hydroquinone is harder to oxidize than the hydroquinone of mitomycin C. The strand cleavage produced by mitomycin C is both more rapid and more extensive than the cleavage caused by mitomycin B. These findings are consistent with the proposed mechanism for the cleavage of DNA by the mitomycins, in which the hydroquinone form of the antibiotic is oxidized in the

presence of oxygen to form the intermediate semiquinone radical. If this oxidation is rendered more difficult - as is the case with mitomycin B - then the damage to DNA resulting from strand scission should be correspondingly lessened.

Experimental

All fluorescence measurements were performed on a G. K. Turner and Associates model 430 spectrofluorometer equipped with a cooling fan to reduce fluctuations in the xenon lamp source. pH measurements for the pKa study were carried out using a Beckman Zeromatic II pH meter and a Fisher 13-639-92 combination electrode. Titrations were performed using a Gilmont Micrometer Buret S1200A of 2 ml capacity. Radioactivity was counted for 20 min in Scinti-Verse on a Searle Analytic Inc. Mark III 6880 Liquid Scintillation counter. For the electrochemical studies a Princeton Applied Research (P. A. R.) Model 9300-9301 polargraphic cell was employed in a three-electrode configuration which included a conventional aqueous saturated calomel reference electrode, a platinum counterelectrode and a P. A. R. Model 9323 hanging mercury drop working electrode. The temperature of the cell was maintained at 37.5° ± 0.2° by circulation of thermostated water. Cyclic voltammetry was done using the P. A. R. Model 173-175-176 configuration, with the resulting curves recorded on an x-y recorder or photographed on an oscilloscope as required by the scan rate

which was varied from 20 mV/sec to 500 V/sec. The pH of each solution was measured before and after each run with an Accumet Model 520 pH meter and a combination glass-SCE electrode.

Materials

Ethidium bromide and catalase were purchased from Sigma Chemical Co. Mitomycin C was from Calbiochem and Kyowa Hakko Kogyo Chemical Co.; mitomycin B was supplied by Kyowa Hakko Kogyo Chemical Co. and American Cyanamid Co. Lederle Labs. λ, PM2, *C. perfringens*, *E. coli*, and calf thymus DNA's were obtained from Dr. A. R. Morgan. Superoxide dismutase was the gift of Dr. Alan Davison.

Fluorescence Assay for Detecting CLC-Sequences

Wavelength calibration of the spectrofluorometer was performed according to the instructions in the instrument manual. One centimeter round cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength 600 nm. The cell compartment was thermostated with water circulated from a bath thermally regulated at 22°. Small aliquots (10 - 20 µl) of the reaction mixtures were added to 2 ml of the assay mixture which was 20 mM potassium phosphate buffer pH 11.6, 0.4 mM EDTA and 0.5 µg/ml in ethidium bromide. For studies involving the pH dependence of the assay solution, a similar assay solution was prepared, which was buffered to pH 7.0. The instrument was blanked with the assay mixture and readings were generally taken on medium

sensitivity (x 100 scale).

The cross-linking assay was performed as follows: a 10 µl aliquot of the reaction mixture was diluted in 2 ml of the assay solution. The fluorescence of this diluted solution was measured. The solution was then heat denatured at 96° for 2 min ± 5 sec, cooled in ice and equilibrated in a constant temperature water bath (22°) for 5 min, after which the fluorescence of the solution was again measured. The extent of covalent cross-linking is given by the ratio of the after-heating fluorescence to the before-heating fluorescence.

General Procedure for the Determination of Cross-Linking and Alkylation of DNA with Reduced Mitomycin B

Mitomycin B was added to the reaction mixtures as an approximately 300 µg/ml aqueous solution; DNA's were also added as aqueous solutions. The reaction solutions were buffered to the appropriate pH with sodium acetate at pH 5.0 or potassium phosphate at pH 6.0, 7.0, 8.7, or 10.3; these buffers were added as 1 M solutions. The reactions were carried out on a 40 - 100 µl scale and had concentrations of 0.9 A_{260} of λ DNA, 0.05 M of the appropriate buffer, 0.6, 1.0, 2.0, or 4.0×10^{-4} M of mitomycin B, and 1.2, 2.0, 3.6, or 5.6×10^{-3} M of sodium borohydride, respectively. At timed intervals 10 µl aliquots of the solution were removed and assayed for the extent of cross-linking as described above. A control solution containing all

components except the mitomycin B was run with each experiment. Assay of the controls showed no cross-linking in each case and established that none of the components in the reaction mixtures interfered with the ethidium fluorescence.

Attempted Reduction of Mitomycin B Using NADH and NADPH

Reactions were carried out on a 40 µl scale and contained λ DNA at 0.9 A_{260} and 0.05 M phosphate buffer pH 7.0. Additional components were 2.0×10^{-4} M mitomycin B and 5.8×10^{-3} M NADH, or 1.8×10^{-4} M mitomycin B and 4.8×10^{-3} M NADPH. 10 µl aliquots were withdrawn at 5 min, 10 min, and 15 min and analyzed for the extent of cross-linking by the ethidium bromide assay; no cross-linking was observed in either case. Control experiments containing no mitomycin B also showed no cross-linking.

Determination of the pKa of Mitomycin B

1 ml of a 7.328×10^{-4} M solution of mitomycin B and 2 ml of boiled, distilled water were titrated with 3.664×10^{-4} M hydrochloric acid. The pH was recorded after 0.9 ml of acid had been added, and thereafter at increments of 0.02 ml until the total volume of acid added was 1.10 ml. When 1.00 ml of acid had been added (one half equivalent), the pH was recorded immediately after the addition of acid and again after 30 sec to confirm that the aziridine ring was not opening on the time scale of the titration. The titration was performed

on duplicate samples.

Procedure for Determining Covalent Cross-Linking of DNA's of Different (G+C) Content by Reduced Mitomycin B

The DNA's used in this study were *C. perfringens* [mol. wt. = 11.4 x 10^6, (G+C) content = 30%], calf thymus [mol. wt. = 9.1 x 10^6, (G+C) content = 40%], and *E. coli* [mol. wt. = 7.7 x 10^5, (G+C) content = 50%]. The molecular weights were determined by sedimentation velocity studies. The composition of the reaction solutions was: DNA at 1.25 A_{260}, 0.05 M phosphate buffer pH 7.0, and mitomycin B at 0.6 or 3.0 x 10^{-4} M, sodium borohydride at 1.2 or 6.2 x 10^{-3} M. Reactions were run at ambient temperature with 10 µl aliquots removed at timed intervals and analyzed for the extent of covalent cross-linking by the ethidium assay.

Cross-Linking by Mitomycin B Without Reduction

The reaction mixture contained λ DNA at 0.95 A_{260}, 0.05 M acetate buffer pH 4.0, and mitomycin B at 2.4 x 10^{-4} M. 10 µl aliquots were withdrawn at timed intervals and analyzed for the extent of cross-linking as described above. A similar reaction mixture containing λ DNA at 0.95 A_{260}, 0.05 M acetate buffer pH 5.0 and mitomycin B at 2.6 x 10^{-4} M showed no cross-linking after 1 hr. Control experiments at each pH with no added mitomycin B showed no acid-induced cross-linking.

Correlation of Loss of Fluorescence with Binding Ratio

Reactions were carried out on a 400 µl scale.

Reaction solutions had concentrations of 1.2 A_{260} of ^{3}H-labelled λ DNA, 0.05 M acetate buffer pH 5.0, mitomycin B at 0.0, 1.8, 5.5, 9.2, 18.3, and 27.5 x 10^{-5} M, and sodium borohydride at 0.0, 5.5, 16.4, 27.3, 54.6 and 82.0 x 10^{-3} M, respectively. The solutions were assayed for loss of fluorescence by the ethidium assay described above, with three 10 µl aliquots removed at timed intervals. The remainder of each solution was dialyzed *vs* 20 mM potassium phosphate pH 11.6, 0.4 mM EDTA. Binding ratios were determined by the procedure of Tomasz[20] except that nucleotide concentrations were determined by radioactive counting. The nucleotide concentration was calculated using an extinction coefficient of 6,600 for DNA at 260 nm. The bound mitomycin B concentration was calculated from the absorbance of the dialyzed reaction solutions at 286 nm using an extinction coefficient of 11,600 for bound mitomycin B. The absorbance at 286 nm was corrected for DNA absorbance at this wavelength using an extinction coefficient of 2,440 for DNA at 286 nm, calculated from the control reaction (mitomycin B concentration = 0).

General Procedure for the Determination of Cleavage of PM2 CCC-DNA with Mitomycin B

Experiments were carried out on a 100 µl scale. Reaction mixtures contained 3.3 or 3.5 x 10^{-4} M mitomycin B, 4.4, 4.6, 5.5, or 5.7 x 10^{-3} M sodium borohydride, PM2 CCC-DNA at 0.6 A_{260}, and 0.05 M phosphate

buffer pH 7.0. 15 µl aliquots were withdrawn at timed intervals and analyzed by the ethidium fluorescence assay described above. A control reaction containing no mitomycin B was run with each experiment. Analysis of the control reaction showed a 75% return of fluorescence after heating which was the same as the value for this particular sample of DNA alone.

Inhibition experiments were performed as above with the addition of the inhibitors listed in Table XIV and Figure 30.

Strand Scission of PM2 CCC-DNA by Covalently Bound Mitomycins

For the study of mitomycin C the following three solutions were prepared on a 500 µl scale: solution A containing PM2 CCC-DNA at 2.4 A_{260}, 0.05 M acetate buffer pH 4.0, and 5.7×10^{-4} M mitomycin C; solution B containing PM2 CCC-DNA at 2.4 A_{260} and 0.05 M acetate buffer pH 4.0; solution C containing 0.05 M acetate buffer pH 4.0 and 5.7×10^{-4} M mitomycin C.

The three solutions were incubated for 1 hr at ambient temperature then dialyzed *vs* 500 ml of 0.05 M phosphate buffer pH 8.0 for 4 hr at 0°. The buffer was then renewed and the dialysis continued for a further 19 hr.

For the study of mitomycin B the following three solutions were prepared on a 500 µl scale: solution A containing PM2 CCC-DNA at 2.4 A_{260}, 0.05 M acetate buffer

Table XIV

Inhibition of Mitomycin B Induced Cleavage of PM2 CCC-DNA

Inhibitor	Time (min) 1	5	10	20
None	76/71[a]	84/83	88/69	89/63
Isopropyl Alcohol (0.25 M)	85/80	85/89	86/88	85/84
Dimethyl Sulfoxide (0.25 M)	52/45	57/53	52/56	48/55
2-Mercaptoethanol (0.25 M)	60/46	65/62	61/56	59/54
Potassium Iodide (0.05 M)	60/51	59/56	56/60	58/58
Sodium Benzoate (0.05 M)	82/74	83/85	84/89	82/83
Cat.[b] (2.3×10^{-7} M)	74/61	75/75	74/71	---
S.D.[c] (1.5×10^{-5} M)	72/63	75/77	79/79	79/73
Cat. (2.4×10^{-7} M) + S.D. (1.5×10^{-5} M)	55/49	51/58	52/55	53/56

[a] Fluorescence before heat denaturation/ Fluorescence after heat denaturation

[b] Cat. Catalase

[c] S.D. Superoxide Dismutase

pH 4.0, and 5.0×10^{-4} M mitomycin B; solution B containing PM2 CCC-DNA at 2.4 A_{260} and 0.05 M acetate buffer pH 4.0; solution C containing 0.05 M acetate buffer pH 4.0 and 5.0×10^{-4} M mitomycin B.

The three solutions were incubated 0.5 hr at ambient temperature, 0.75 hr at 37°, and a further 2 hr at ambient temperature, then dialyzed *vs* 500 ml of 0.05 M phosphate buffer pH 8.0 for 5 hr at 0°. The buffer was then renewed and the dialysis continued for a further 17 hr.

In both studies, scission reactions with dialyzed solutions A and B were performed as described above at ambient temperature in 0.05 M phosphate buffer pH 7.0 containing approximately 1.1 A_{260} of the DNA and approximately 6.0×10^{-3} M sodium borohydride. Additional experiments with each solution A also contained 2.4×10^{-7} M catalase, 1.5×10^{-5} M superoxide dismutase, or 0.25 M isopropyl alcohol.

A control experiment was performed to show that all the free mitomycin in each case was removed by the dialysis. A 100 µl solution containing 62 µl of dialyzed solution C, 0.05 M phosphate buffer pH 7.0, native PM2 CCC-DNA at 1.0 A_{260}, and approximately 6.0×10^{-3} M sodium borohydride was incubated at ambient temperature. Ethidium assay analysis of 15 µl aliquots withdrawn at timed intervals showed no single strand scission of the DNA.

Effect of Intercalated Ethidium on the Mitomycin B Induced Single Strand Scission and Cross-Linking of PM2 CCC-DNA

Reactions were carried out at ambient temperature on a 200 µl scale. Solutions contained PM2 CCC-DNA at 0.6 A_{260}, 0.05 M phosphate buffer pH 7.0, and mitomycin B at 1.0×10^{-4} M. Additional components were sodium borohydride at 2.5, 2.2, 2.2, and 2.9×10^{-3} M, and ethidium bromide at 0, 6.3, 25, and 63×10^{-6} M, respectively. The solutions were prepared by allowing the ethidium to interact with the buffered DNA for 5 min prior to the addition of the mitomycin B and the sodium borohydride. After the preparation of the solutions, 20 µl aliquots were withdrawn at timed intervals and analyzed by the ethidium assay for the extent of strand scission and for indications of cross-linking. A control experiment containing no mitomycin B showed no strand scission. Solutions were stoppered and protected from light to prevent possible cleavage of the DNA by the ethidium itself.[56]

Effect of Intercalated Ethidium on the Mitomycin C Induced Single Strand Scission and Cross-Linking of PM2 CCC-DNA

Experiments analogous to the above were performed using mitomycin C. Reaction mixtures contained PM2 CCC-DNA at 0.6 A_{260}, 0.05 M phosphate buffer pH 7.0, and mitomycin C at 1.5×10^{-4} M. Additional components were sodium borohydride at 3.5, 3.3, 3.3, and 3.4×10^{-3} M, and ethidium bromide at 0, 6.3, 25, and 63×10^{-6} M, respectively.

Acid-Catalyzed Opening of the Aziridine Ring of Mitomycin B. Preparation of 55

Hydrolysis of mitomycin B was carried out by a modification of the procedure of Stevens *et al.*[22a] 8.75 mg of mitomycin B was dissolved in 2 ml of 0.05 M hydrochloric acid and stirred at ambient temperature for 75 min. The solution was then made basic (pH = 9) with anhydrous potassium carbonate and extracted with ether. Removal of the ether left a residue which was dried *in vacuo* then dissolved in a small amount of ether and precipitated by the addition of *n*-pentane as 2.92 mg (32% yield) of a yellow-orange solid m.p. 70-72°. Thin-layer chromatography on silica with isopropyl alcohol:benzene (50:50 v/v) as eluant showed a single spot. The derivative 55 is soluble in chloroform, ether, and water.

The ir spectrum ν_{max} ($CHCl_3$): 2960, 2920 (CH_3 sym. str.); 2860; 1715 (carbamate C=O); 1660, 1645 (quinone C=O); 1595; 1450; 1395; 1250 (=C-O-C antisym. str.); 1085; 1000 (=C-O-C sym. str.) cm^{-1}.

The absorbances λ_{max} (H_2O): 236 (log ε 4.16), 286 (log ε 4.06), 355 (log ε 3.26), 442 (log ε 2.89) nm.

Determination of Cross-Linking of DNA with Reduced Ring-Opened Mitomycin B 55

Experiments were performed as described above for mitomycin B. Reaction solutions had concentrations of 0.6 A_{260} of λ DNA, 0.05 M phosphate buffer pH 7.0, 3.3×10^{-4} M of derivative 55, and 4.2×10^{-3} M sodium

borohydride.

Determination of Interaction of Reduced Mitomycin Derivative 55 with PM2 CCC-DNA

Experiments were performed as described above for mitomycin B. Reaction solutions had concentrations of 0.6 A_{260} of PM2 CCC-DNA, 0.05 M phosphate buffer pH 7.0, 3.3×10^{-4} M of derivative 55, and 4.9×10^{-3} M sodium borohydride. Additional experiments also contained 0.25 M isopropyl alcohol. Control experiments lacking the mitomycin derivative showed a 90% return of fluorescence after heating (the same as DNA alone) indicating that this sample of PM2 CCC-DNA was nicked to the extent of about 10%.[49]

Electroanalytical Procedures

A stock solution of 1.3×10^{-3} M mitomycin B was prepared and protected from light. Sample solutions were prepared in the cell to give 3.3×10^{-4} M mitomycin B, 0.1 M phosphate buffer at the appropriate pH in the range 6.0 to 8.5, and 0.1 M potassium chloride as supporting electrolyte. Mitomycin B derivative 55 was treated in a similar manner beginning with an 8.4×10^{-4} M stock solution. All solutions were deaerated with purified nitrogen gas for 10 min prior to each run and blanketed with it during each run. All potentials were taken relative to an aqueous saturated calomel electrode.

BIBLIOGRAPHY

1. T. Hata, Y. Sano, R. Sugawara, A. Matsumae, K. Kanamori, T. Shima, and T. Hoshi, J. Antibiot. (Tokyo), Ser. A, 9, 141 (1956).

2. J.S. Webb, D.B. Cosulich, J.H. Mowat, J.B. Patrick, R.W. Broschard, W.E. Meyer, R.P. Williams, C.F. Wolf, W. Fulmor, C. Pidacks, and J.E. Lancaster, J. Amer. Chem. Soc., 84, 3185, 3187 (1962).

3. A. Tulinsky, J. Amer. Chem. Soc., 84, 3188 (1962).

4. R. Yahashi and I. Matsubara, J. Antibiot., 29, 104 (1976).

5. E.J. Kirsch, in "Mitomycins" in "Antibiotics II", edited by D. Gottlieb and P.D. Shaw, Springer-Verlag Publishers, New York, 1967, p. 66.

6. (a) C.C.J. Culvenor, A.T. Dann, and A.T. Dick, Nature, 195, 570 (1962);
(b) C.C.J. Culvenor, D.T. Downing, J.A. Edgar, and M.V. Jago, Ann. N.Y. Acad. Sci., 163, 837 (1969);
(c) A.J. Aasen and C.C.J. Culvenor, Aust. J. Chem., 22, 2657 (1969).

7. W. Szybalski and V.N. Iyer, in "The Mitomycins and Porfiromycins" in "Antibiotics I", edited by D. Gottlieb and P.D. Shaw, Springer-Verlag Publishers, New York, 1967, p. 211.

8. S.K. Carter, Cancer Chemother. Rep. Part 3, 1, 99 (1968).

9. G.E. Moore, I.D.J. Bross, R. Ausman, S. Nadler, R. Jones Jr., N. Slack, and A.A. Rimm, Cancer Chemother. Rep., 52, 675 (1968).

10. "Merck Index", edited by P.G. Stecher, eighth edition, Merck and Co. Publishers, New Jersey, 1968, p. 698.

11. H. Kersten and W. Kersten, in "Inhibitors of Nucleic Acid Synthesis" in "Molecular Biology, Biochemistry, and Biophysics", vol. 18, Springer-Verlag Publishers, New York, 1974, p. 4.

12. J.W. Lown, A. Begleiter, D. Johnson and A.R. Morgan, Can. J. Biochem., 54, 110 (1976).

13. A. Begleiter, "Studies Related to the Antitumor Antibiotic Mitomycin C and Analogs", Ph.D. thesis, University of Alberta, 1976.

14. K. Okamoto, J.A. Mudd, J. Mangan, W. Huang, T.V. Subbaiah, and J. Marmur, J. Mol. Biol., 34, 413 (1968).

15. S. Shiba, A. Terawaki, T. Taguchi, and J. Kawamata, Nature, 183, 1056 (1959).

16. H. Kersten, B. Schnieders, G. Leopold, and W. Kersten, *Biochim. Biophys. Acta*, 108, 619 (1965).

17. K. Lapis and W. Bernhard, Cancer Research, 25, 628 (1965).

18. S. Tsukamura and M. Tsukamura, Japan J. Microbiol., 6, 53 (1962).

19. (a) V.N. Iyer and W. Szybalski, Proc. Nat. Acad. Sci. U.S.A., 50, 355 (1963);
(b) V.N. Iyer and W. Szybalski, Science, 145, 55 (1964);
(c) W. Szybalski and V.N. Iyer, Federation Proc., 23, 946 (1964).

20. A.J. Lin, L.A. Cosby, and A.C. Sartorelli, Cancer Chemother. Rep., Part 2, 4, 23 (1974).

21. M. Eigen and D. Porschke, J. Mol. Biol., 53, 123 (1970).

22. (a) C.L. Stevens, K.G. Taylor, M.E. Munk, W.S. Marshall, K. Noll, G.D. Shah, L.G. Shaw, and K. Uzu, J. Med. Chem., 8, 1 (1964);
(b) S. Miyamura, N. Shigeno, M. Matsui, S. Wakaki, and K. Uzu, J. Antibiot. (Tokyo), Ser. A, 20, 72 (1967);
(c) M. Matsui, Y. Yamada, K. Uzu, and T. Hirata, J. Antibiot. (Tokyo), 21, 189 (1968);
(d) S. Kinoshita, K. Uzu, K. Nakano, M. Shimizu, T. Takahashi, and M. Matsui, J. Med. Chem., 14, 103 (1971).

23. J.B. Patrick, R.P. Williams, W.E. Meyer, W. Fulmor, D.B. Cosulich, R.W. Broschard, and J.S. Webb, J. Amer. Chem. Soc., 86, 1889 (1964).

24. M. Tomasz, C.M. Mercado, J. Olson, and N. Chatterjie, Biochemistry, 13, 4878 (1974).

25. M.N. Lipsett and A. Weissbach, Biochemistry, 4, 206 (1965).

26. P.D. Lawley and P. Brookes, Biochem. J., 89, 127 (1963).

27. A. Weissbach and A. Lisio, Biochemistry, 4, 196 (1965).

28. M. Tomasz, *Biochim. Biophys. Acta*, 213, 288 (1970).

29. (a) E. Reich, A.J. Shatkin, and E.L. Tatum, *Biochim. Biophys. Acta*, 45, 608 (1960);
(b) E. Reich, A.J. Shatkin, and E.L. Tatum, *Biochim. Biophys. Acta*, 53, 132 (1961);
(c) H. Kersten and H.M. Rauen, Nature, 190, 1195 (1961);
(d) S. Wakaki, Cancer Chemother. Rep., 13, 79 (1961).

30. J.D. Albright and H.R. Snyder, J. Amer, Chem. Soc., 81, 2239 (1959).

31. R.J. Sundberg, "The Chemistry of Indoles", Academic Press, New York, 1970, p. 93.

32. E. Leete, J. Amer. Chem. Soc., 81, 6023 (1959).

33. (a) S. Glasstone and D. Lewis, "Elements of Physical Chemistry", second edition, D. Van Nostrand Co. Inc., Princeton, 1960, p. 627;
(b) K.B. Wiberg, "Physical Organic Chemistry", Wiley and Sons, Inc., New York, 1964, p. 377.

34. T.A. Geissman and A. Armen, J. Amer. Chem. Soc., 74 3916 (1952).

35. H. Hsiung, D. Johnson, and J.W. Lown, unpublished results.

36. H. Murakami, J. Theoret. Biol., 10, 236 (1966).

37. C.H. Rochester, in "The Chemistry of the Hydroxyl Group, part 1", edited by S. Patai, Interscience Publishers, London, 1971, p. 369.

38. (a) C. Schotten, *Ber.*, 17, 2544 (1884);
(b) E. Baumann, *Ber.*, 19, 3218 (1886).

39. H.R. Snyder and E.L. Eliel, J. Amer. Chem. Soc., 70, 1857 (1948).

40. (a) L.F. Fieser, "Experiments in Organic Chemistry", third edition, D. Heath and Co., Boston (1957), p. 289;
(b) *ibid.*, p. 291.

41. H.C. Brown, S. Johnson, and H. Podall, J. Amer. Chem. Soc., 76, 5556 (1954).

42. E. Fischer and O. Hess, *Ber.*, 17, 559 (1884).

43. H.R. Snyder and E.L. Eliel, J. Amer. Chem. Soc., 70, 1703 (1948).

44. W. Schindler, *Helv. Chim. Acta*, 40, 2156 (1957).

45. J.A. Eenkhoorn, S.O. de Silva, and V. Snieckus, Can. J. Chem., 51, 792 (1973).

46. K. Eiter and O. Svierak, *Monatsh.*, 83, 1453 (1952); Chem. Abstr., 48, 693c.

47. J. Madinaveitia, J. Chem. Soc., 1927 (1937).

48. J.B. Le Pecq and C. Paoletti, J. Mol. Biol., 27, 87 (1967).

49. A.R. Morgan and V. Paetkau, Can. J. Biochem., 50, 210 (1972).

50. (a) A.R. Morgan and D.E. Pulleyblank, Biochem. Biophys. Res. Comm., 61, 396 (1974);
(b) J. Burnotte and W.G. Verley, *Biochim. Biophys. Acta*, 269, 370 (1972).

51. R. Cone, S.K. Hasan, J.W. Lown, and A.R. Morgan, Can. J. Biochem., 54, 219 (1976).

52. K. Handa and S. Sato, *Gann*, 66, 43 (1975).

53. M. Tomasz, Chem. Biol. Interactions, 13, 89 (1976).

54. J. Bearden Jr. and C.W. Haidle, Biochem. Biophys. Res. Comm., 65, 371 (1975).

55. G.M. Rao, A. Begleiter, J.W. Lown, and J.A. Plambeck, submitted to J. Electrochem. Soc.

56. D.E. Pulleyblank and A.R. Morgan, Can. J. Biochem, submitted for publication.